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PRINCIPAL INVESTIGATOR: Jiaoti Huang, M.D., Ph.D.

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6. AUTHOR(S)
Jiaoti Huang, M.D., Ph.D.

E-Mail: JiaotiHuang@mednet.ucla.edu

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University of California Los Angeles
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14. ABSTRACT
Purpose: The goal of the project is to determine the function of neuroendocrine (NE) cells in the initiation and progression of human prostate cancer
Scope: 1) Use a pten null mouse prostate cancer model to determine if ablation of NE cells by selective expression of a toxin in these cells can delay or prevent tumor initiation and/or progression. 2) Use a human tissue recombination model to determine if depletion of NE cells from human epithelial cells can retard the initiation and progression of the recombinant tumor. 3). Demonstrating the origin and molecular basis of human small cell carcinoma
Major findings: 1) We have established a robust protocol to procure fresh human prostate tissue and isolate subpopulations of prostatic epithelial cells; 2) We have identified the appropriate combinations of cell surface markers for the isolation of NE cells; 3) We have demonstrated that neuroendocrine cells are not required for tumor initiation in a pten knockout mouse prostate cancer model; 4) With the tissue recombination model, tumors can be generated using the neuroendocrine cell-depleted basal cells, suggesting that neuroendocrine cells are incapable of initiating small cell neuroendocrine carcinoma in the tissue recombination model.

15. SUBJECT TERMS
Prostate cancer, initiation, progression, neuroendocrine cells, animal model

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1. Introduction:

Despite years of study by clinicians and scientists around the world, there are still many unanswered questions in prostate cancer. A fundamental and clinically important issue is why prostate cancer responds to hormonal therapy initially but becomes resistant eventually in nearly all patients [1]. Prostate cancer is histologically heterogeneous consisting of luminal type tumor cells and a small component of neuroendocrine (NE) cells [2]. Unlike luminal type tumor cells that express androgen receptor (AR) and depend on androgen for proliferation, NE cells lack AR and are androgen-independent [3]. Hormonal therapy, while inhibiting luminal tumor cells, increases the number of NE cells in prostate cancer which is evident in recurrent castration-resistant prostate cancer[4]. In some patients, the recurrent tumor is composed of pure NE cells and is classified as small cell neuroendocrine carcinoma (SCNC) [5]. We hypothesize that NE cells play important roles in the initiation and progression of PC. We also hypothesize that they are the cells of origin for SCNC and p53 is the molecular target. This research proposal has the following specific aims:

1) To determine if NE cells are required for tumor initiation and/or progression in a mouse PC model;
2) To determine if NE cells are required for tumor initiation and/or progression in a human PC model;
3) Cell of origin and the molecular targets of prostatic small cell neuroendocrine carcinoma.

2. Keywords:

Prostate cancer, initiation, progression, castration resistance, neuroendocrine cells, androgen receptor, cell of origin

3. Overall project summary:

Research accomplishments associated with Task 1: In this task, we will generate *pten*$^{loxP/loxP}$/pb-Cre/CR2-toxin+ mice by breeding pten conditional knockout mice with CR2-toxin mice. We will then observe tumor development and whether the mice develop castration-resistant tumors after castration (Time frame: Months 1 – 36)

1a: Breeding and genotyping (Time frame: Months 1 – 24)

Our goal is to determine the function of neuroendocrine cells in the initiation and progression of prostate cancer. Our approach is to compare the mice with or without NE cells in the prostate to observe prostate cancer formation. The hypothesis is that in the male mice, the toxin will be expressed in prostate neuroendocrine cells because of the selective activity of CR2 promoter in such cells [6, 7], resulting in ablation of the neuroendocrine cells. This will give us an opportunity to definitively determine the function of neuroendocrine cells in prostate cancer.

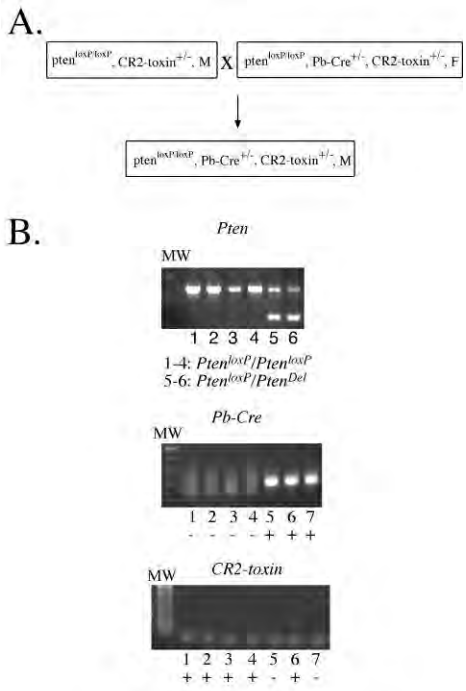

Figure 1. Genotyping strategies to identify mouse of desired genetic composition through PCR.

We have successfully established genotyping protocol for identifying mice that are of the desired genotype. As can be seen from Figure 1B, mice with prostate deletion of *Pten* can be identified through PCR by 3 primers: WT forward 5'TCCCAGAGTTCATACCAGGA3', WT reverse 5'GCAATGGCCAGTACTAGTGAAC3' and an internal primer 5'AATCTGTGCATGAAGGGAAC3'. For *Probasin-Cre* detection, we used the following primers: 5'CAAAACAGGTAGTTATTCGG3' and 5'CGTATAGCCGAAATTGCCAG3'. For detection of *CR2-toxin*, we used two rounds of PCR. In the first round, the following primers were used: 5'cttaacgctttcgcctgttc3' and 5'tcgtaccacgggactaaacc3'. The product of the first PCR reaction was diluted 20 fold and used as the input for the 2nd round of PCR with the primers: 5'gctctctggaaaagctggag3', 5'agggaaggctgagcactaca3'.

We have found a productive strategy to derive mice of desired genotypes after we realized that male mouse with prostate deletion of *Pten* is most likely not fertile. We therefore bred male mouse carrying the floxed *Pten* allele to the female mouse carrying both the floxed *Pten* and *Cre* gene as outlined in Figure 1A.

This task has therefore been successfully completed.

1b: Observing the development of primary tumors (Time frame: Months 12 – 24)

Although pten-/- model will be used to demonstrate the function of neuroendocrine cells, an important control is the TRAMP (transgenic adenocarcinoma of the mouse prostate) mouse prostate cancer model. Prostate tumors in TRAMP mice are composed exclusively of neuroendocrine cells. Therefore this model is a perfect control for us to demonstrate if expression of toxin can abolish the neuroendocrine cells and delay or prevent the development of the tumor as a result.

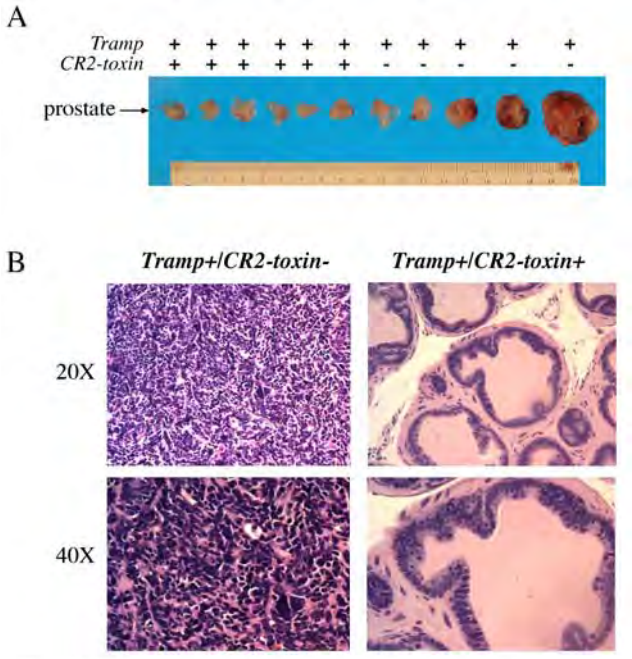

Figure 2. CR2-toxin expression blocks the small cell carcinoma development in mice induced by TRAMP expression. A. Prostates from mice with indicated genotypes. B. H&E staining of the prostate pathology with Tramp and CR2-toxin expression.

Therefore, we have crossed TRAMP mice with CR2-toxin mice and generated TRAMP mice with or without the expression of CR2-toxin. As shown in Figure 2A, mice without CR2-toxin expression develops aggressive tumor whose pathology is consistent with small cell neuroendocrine carcinoma (Figure 2B). However, in mice with co-expression of CR2-toxin, there is no visible tumor formation upon dissection, which is supported by histological examination (Figure 2B). These results demonstrate that our approach to abolish neuroendocrine cells in the prostate with CR2 promoter-driven diphtheria toxin is a valid strategy, which will help us to definitively determine if neuroendocrine cells play a role in prostate cancer development and progression.

Table 1. A collection of mice of various ages.

Pten$^{loxP/loxP}$/Pb-Cre Age (wks)	*CR2-toxin+*	*CR2-toxin-*
54	2	1
43	1	2
38	2	6
33	2	2
29	2	2
25	3	2
21	3	2
total	15	17

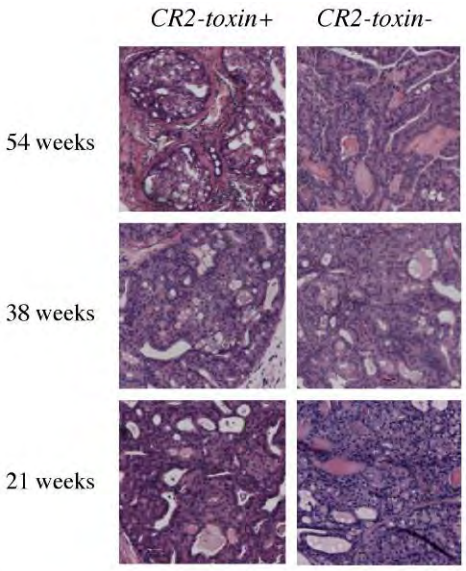

Figure 3. Morphological comparisons among mice with or without diptheria toxin driven by CR2 promoter.

As outlined in Table 1, we have generated 32 conditional pten knockout mice with the desired genotypes of various ages. There were 15 mice carrying the diphtheria toxin, likely devoid of neuroendocrine cells as the toxin will kill the target cells. There were 17 mice without the toxin genes. The prostates of those mice were dissected and whole mount H&E analyses were performed on all of those samples. As shown in Figure 3, a sampling of histological analyses of those mice did not show a difference in the morphology of those prostates which were uniformly cancerous.

1c: Castration and observation of the development of castration-resistant tumors (Time frame: Months 24-36)

The goal of this aim is to determine if neuroendocrine cells contribute to the appearance of castration-resistant prostate cancer after hormonal therapy. Most of the animals with the appropriate genotypes have been used for aim 1b. However, we collected 4 mice that were castrated at age

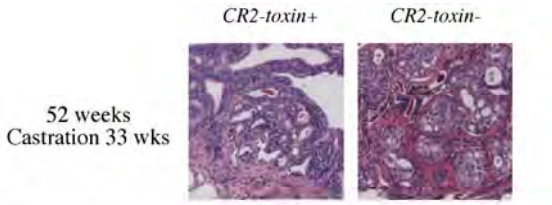

Figure 4. Morphological comparisons among castrated mice with or without CR2-toxin expression.

of 19 weeks and continued for 33 weeks. There might be an interesting difference between the group carrying the toxin gene vs. the mouse that did not have the toxin gene. It appears that both groups have a smaller prostate due to castration, and the morphology of the prostate is of PIN, rather than of cancer. However, the toxin group seems to have more localized PIN rather than a more developed PIN pathology. Due to the limited number of mice of the castration group, we cannot make a firm conclusion about whether the neuroendocrine cells might play a role in castration-resistant growth of the *Pten*-loss induced prostate cancer.

While we were trying to expand the mice for a larger study, we discovered that our mice have lost the expression of *CR2-toxin* transgene before we are able to complete the entire project. Loss of transgene expression is not an infrequent problem encountered by researchers. We reported this problem to DOD and requested a no-cost extension last year. We tried to solve this problem and to continue collecting mice with the desired the genotype of expressing *CR2-toxin* in mice with prostate deletion of *Pten* but did not succeed. We also tried to request the transgenic mice from University of Texas Southwestern medical Center that provided the mice to us originally or to purchase from other companies. Unfortunately, neither University of Texas Southwestern medical Center nor other companies have this particular strain any more.

Research accomplishments associated with Task 2: In this task, we will procure fresh human prostate cancer tissue, separate tumor from benign prostate, separate epithelial cells into NE and non-NE cells, and perform tissue regeneration experiments to determine if NE cells are essential in tumor initiation and progression

2a. Procurement of fresh human prostate cancer tissue, separate tumor from benign prostate, separate tumor cells into NE and non-NE tumor cells (Time frame: Months 1 – 36)

We have established a robust system for the procurement of fresh prostate tissue, involving close collaborations among urologists, pathologists, Pathologist Assistants, technical staff from UCLA's Translational Pathology Core Laboratory (TPCL) and basic researchers [8]. There is a seamless workflow starting at the time when the prostate is removed from the patients and including rapid transportation of the specimen to pathology, gross examination of the prostate, procurement of tissue for research, diagnosis of the procured tissue, separation of the tissue into benign prostate and prostate cancer, preparation of single cell suspension and flow cytometric separation of sub-population of epithelial cells based on cell surface markers. We have published a high profile article describing the technology developed by our group [8].

We explored the utility of several candidate cell-surface markers for the isolation/purification of an enriched subset of neuroendocrine cells using Fluorescence Activated Cell Sorting (FACS) on dissociated human prostate tissue preparations. After significant attempts and optimization, we identified the antigen CD56/NCAM (Neural Cell Adhesion Molecule) as the most robust marker for NE cells in primary human

prostate. Our gating strategy was based on a negative depletion for the hematopoietic cell-surface marker CD45, positive enrichment for the epithelial antigens EpCAM (Trop1) or Trop2, and further separation into CD56+ (NE-enriched) and CD56- (NE-depleted) cells. Quantitative PCR analysis for classical NE genes including chromogranin A and Neuron-Specific Enolase (NSE) demonstrated significant enrichment for NE-specific transcripts in the CD56+ fraction compared to the CD56- subset.

2b. Tissue regeneration experiment to determine if NE cells are involved in tumor initiation (Time frame: Months 1 – 24)

In our attempt to determine the role of distinct epithelial lineages in prostate cancer development and progression, we have generated and characterized a model of human prostate cancer initiating in naïve human prostate epithelial cells transduced with the oncogenes Myc and myristoylated AKT (myrAKT). Transduction of Trop2+CD49f^{lo} CD26+ luminal cells and Trop2+ CD56+ neuroendocrine cells did not result in any detectable tumors. In contrast, Trop2+ CD49f^{hi} CD26- basal cells were efficient targets for cancer initiation.

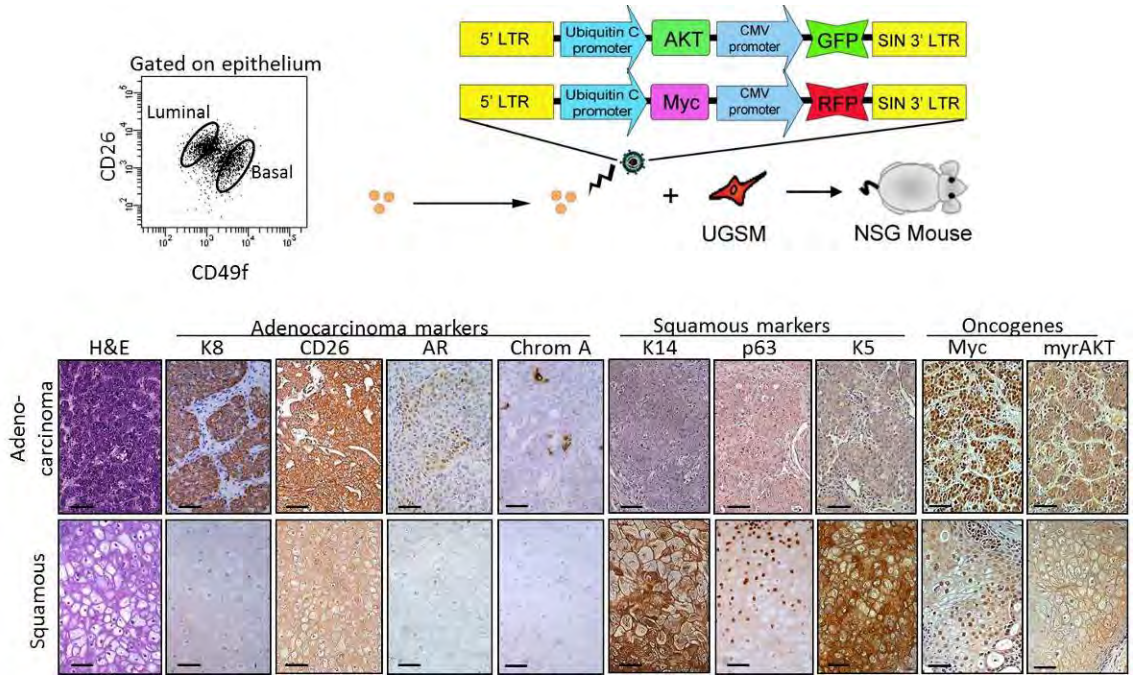

Figure 5. Schematic of naïve human prostate in vivo transformation. Top: CD45-Trop2+ epithelial cells were sorted based on CD49f and CD26 into CD49f^{hi}CD26^{-} basal-enriched and CD49f^{lo}CD26^{+} luminal-enriched subsets, transduced with lentivirus carrying Myc, myrAKT or both, combined with UGSM cells and transplanted into NSG mice. Bottom: Representative adenocarcinoma and squamous regions are identified based on staining for H&E and antibodies against luminal markers Keratin 8 (K8), CD26 and androgen receptor (AR), the neuroendocrine marker chromogranin A, and basal/squamous markers Keratin 14 (K14), p63 and Keratin 5 (K5), and oncogenes Myc and myrAKT/pAKT. Scale bars, 100 µm.

Interestingly, we have found that primary tumors derived from Myc/myrAKT-transformed basal cells exhibit a heterogeneous or mixed tumor response, containing

features of both adenocarcinoma and squamous cell carcinoma. Cells bearing both histological phenotypes are human in origin and express the oncogenes Myc and AKT. However, several markers are preferentially expressed in one or the other histological subtype. Expression of luminal-type markers Keratin 8, CD26 and Androgen receptor, and the neuroendocrine marker chromogranin A are found exclusively in adenocarcinoma tumor foci. In contrast, basal cell marker Keratin 5, Keratin 14, and p63 are exclusively expressed in squamous tumor foci.

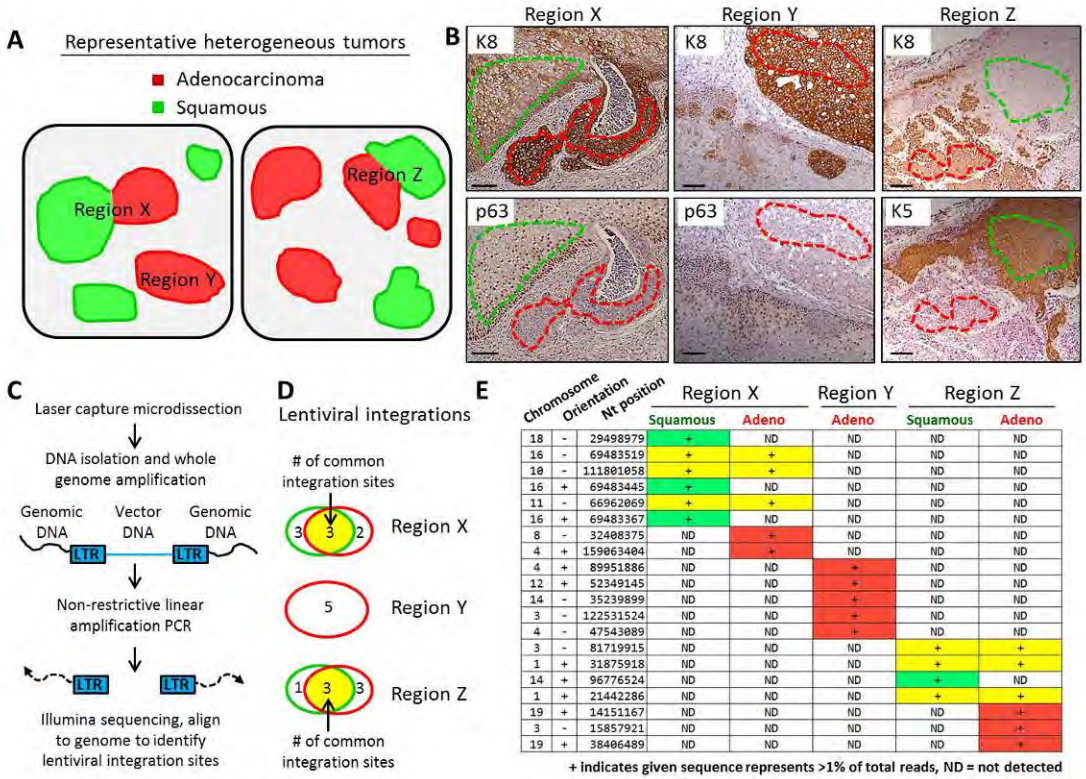

Figure 6. Distinct histological variants in heterogeneous tumors can share a clonal origin. (A) Schematic of two different heterogeneous tumors containing adenocarcinoma, squamous or both phenotypes. Regions X, Y and Z were further studied for lentiviral integration site analysis. (B) Laser capture microdissection was performed on individual glands containing both squamous and adenocarcinoma phenotypes. Representative regions X, Y and Z are shown with serial tissue sections stained with K8 to highlight adenocarcinoma and either p63 or K5 to highlight squamous regions. Dotted lines indicate region excised using laser capture microdissection. Scale bars, 100 µm. (C) Schematic of lentiviral integration site analysis. LTR: long terminal repeat (viral DNA), PCR: polymerase chain reaction. (D) Venn diagrams depict shared lentiviral integration sites in DNA isolated and amplified from neighboring adenocarcinoma (red) and squamous (green) phenotypes (region X), distinct adenocarcinoma gland (region Y), and additional neighboring adenocarcinoma and squamous phenotypes (region Z). (E) Table lists all unique integration sites (IS) with genomic location identifiers (chromosome, orientation, nucleotide position) representing at least 1% of total reads (indicated by +) in each sample. Highlighted rows in yellow represent shared IS between distinct histological phenotypes in the same region, rows in red indicate IS unique to adenocarcinoma, and green represent IS unique to squamous. Note: different regions (X, Y, Z) do not share any IS.

In addition to separate adenocarcinoma and squamous tumor foci, we also found mixed foci containing cells with both histological phenotypes. Since tumors were

initiated by lentiviral delivery of oncogenes into basal cells, the viral sequence randomly integrates into the genome of the target cell and all of its progeny. We isolated DNA from neighboring adenocarcinoma and squamous cells within a mixed tumor foci, and performed PCR extending from the viral DNA into the host genome containing the lentiviral integration site. We then performed deep sequencing and aligned reads to the genome. Our analysis revealed that both histological phenotypes are derived from a common clonal cell of origin.

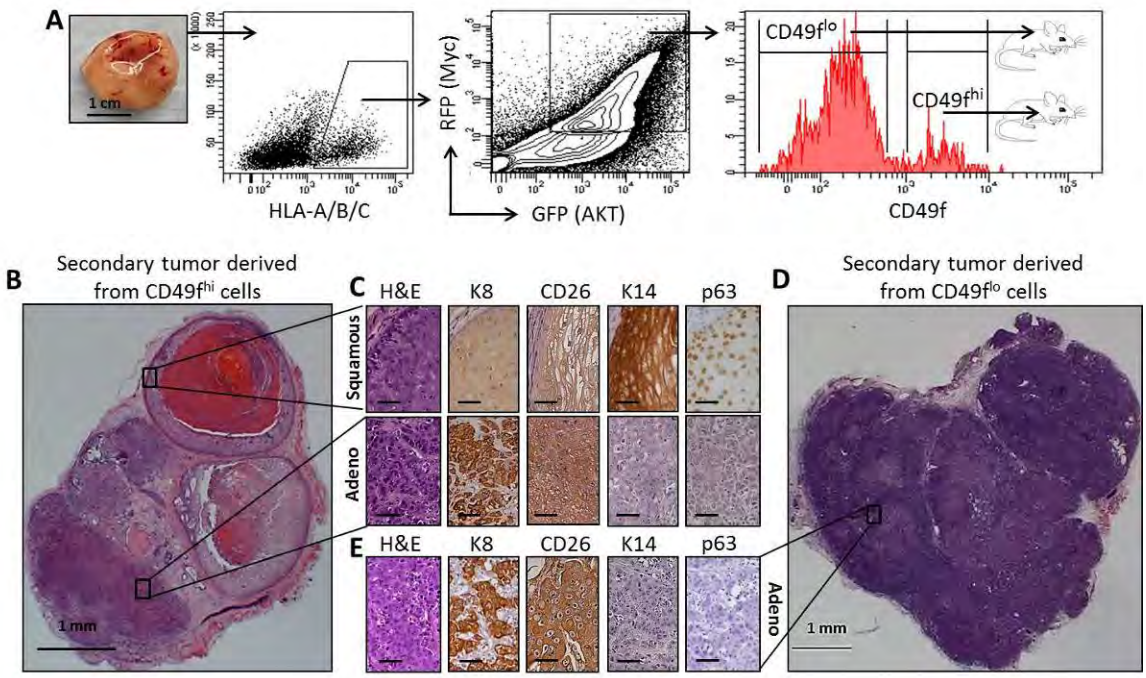

Figure 7. Two phenotypic cell populations can propagate tumors. (A) Tumors initiated from CD49f^{hi} cells expressing Myc and myrAKT are dissociated to single cells, stained with a pan-HLA-A/B/C human antibody and gated based on HLA+, GFP+/RFP+ from lentivirus carrying oncogenes myrAKT (GFP) and Myc (RFP), and further sorted into CD49f^{hi} and CD49f^{lo} subsets. Isolated subsets are transplanted back into recipient mice and harvested 6-12 weeks later. (B) H&E-stained overview of a representative secondary tumor from 10,000 isolated CD49f^{hi} tumor cells. (C) Both squamous and adenocarcinoma (Adeno) phenotypes are represented in secondary tumors as distinguished by stains for H&E, K8, CD26, K14 and p63. Scale bars, 50 µm. (D) H&E-stained overview of a representative secondary tumor generated from 10,000 isolated CD49f^{lo} tumor cells. (E) Only the adenocarcinoma phenotype is observed as evidenced by stains for H&E, K8, CD26, K14 and p63. Scale bars, 50 µm.

To determine which cell-types are capable of propagating tumors, we utilized the antigen CD49f. We first showed that CD49f was expressed highly in the basal layers of squamous tumor cells, but not in adenocarcinoma. In contrast, adenocarcinoma cells express low levels of CD49f. We then fractionated CD49f^{hi} and CD49f^{lo} cells and transplanted both into recipient mice. Both phenotypic populations were competent to propagate tumors, however the histologies represented in the tumors differed. CD49f^{hi} cells could propagate heterogeneous mixed tumors, while CD49f^{lo} cells could only transplant adenocarcinoma.

2c. Serial transplantation and tissue regeneration to determine if NE cells are involved in tumor progression

Regardless of the role of NE cells in the initiation of prostate cancer, NE cells are continually found in tumors suggesting a role in tumor progression and maintenance [9]. As described above, we can establish aggressive primary human prostate cancer using lentiviral Myc and AKT transduced primary cells combined with UGSM cells in vivo into immune-deficient mice.

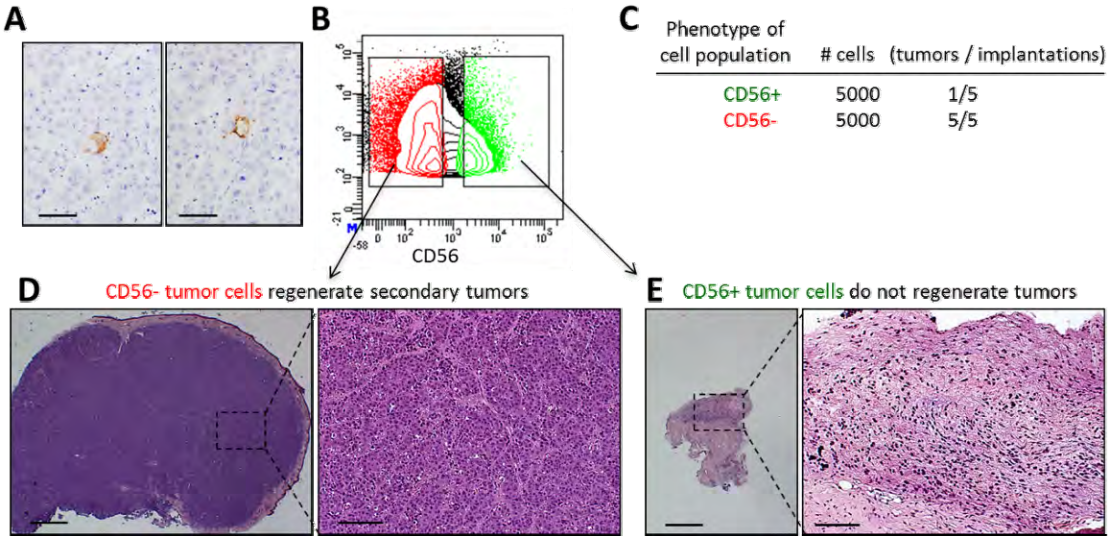

Figure 8. Tumor-propagating cells do not express the neuroendocrine cell marker CD56. CD49f^{lo} cells from primary regenerated tumors were transplanted into recipient mice to establish secondary tumors. (**A**) Secondary tumors were stained for chromogranin A to detect rare neuroendocrine-like cells. Scale bars, 50 µm. (**B**) Secondary tumor cells were sorted based on HLA+ RFP+ GFP+ and further divided into CD56+/- fractions and transplanted into recipient mice. (**C**) Tumors formed consistently from the transplantation of 5000 CD56- cells but only one tumor formed out of five transplantations of 5000 CD56+ cells. (**D**) A representative secondary tumor derived from CD56- tumor cells is stained for H&E. (**E**) A representative graft comprised of mesenchymal cells without a detectable tumor is shown, stained for H&E. Scale bars, 1 mm. Magnified image, 200 µm.

While high levels of Myc alone or AKT alone were not sufficient to drive full progression to cancer, the combination synergized to initiate large highly-proliferative tumors. Dissociated tumor cells were capable of propagating adenocarcinoma upon transplantation into mice. Tumors maintained a phenotype that was Keratin 8+ and p63- indicating an acinar-type or luminal-like cell. Importantly, staining for chromogranin A indicated continued presence of NE cells in tumors. Therefore, we separated out the CD56+ and CD56- fraction from aggressive prostate tumors initiated by Myc and AKT, and transplanted each subset into mice. After 12 weeks, only the CD56- (NE-depleted) fraction could initiate tumors, demonstrating that in this model, NE cells may not be required for tumor propagation.

Research accomplishments associated with Task 3: In this task, we will procure fresh human prostate cancer tissue, separate tumor from benign prostate, separate

epithelial cells into NE and non-NE cells, and perform tissue regeneration experiment to determine if SV40 T antigen induces SCNC in NE cells and if p53 is the molecular targets

3a: Same as 2a

3b. Tissue regeneration experiment to determine if NE cells are the cells of origin for SCNC (Time frame: Months 1 – 24)

Dissociated naïve benign human prostate tissue was separated by FACS into CD56+ (NE-enriched) and CD56- (NE-depleted) fractions and then transduced with lentivirus carrying the SV40 Large T-antigen. Transduced cells were combined with UGSM and transplanted into mice in vivo. Dissociated cells from two different patients were tested and no growths were established from either the CD56+ or CD56- fraction. These findings suggest two possibilities. First, the quality of the tissue from these patients may not have been sufficient for continued growth of the cells in the new hosts. Working with primary human tissue can be challenging as the length of time that tissues are kept without a blood supply before being utilized for research can vary greatly. The second possibility is that the SV40 T-antigen is toxic to naïve benign primary human prostate cells when introduced through lentiviral transduction.

3c. Tissue regeneration experiment to determine if p53 is the molecular target for SCNC (Time frame: Months 12 – 36)

We have successfully established tissue regeneration system from fresh human prostate epithelial cells immediately following prostatectomy. This is a generally robust experimental model but there are still challenges. Unfortunately, we have had some problems with our human prostate transformation system in the past year. We were unable to regenerate human structures using the tissue provided to us through the pathology core following radical prostatectomy. The main problem is that many of the surgeries are finished late in the day. Since such tissues need to be processed by pathology personnel for diagnosis first before any tissue is taken for research, the availability of pathology personnel is crucial. When the prostate arrives in pathology late in the day and cannot be processed same day, the prostate is held in media overnight prior to processing the following day. Although this is perfectly fine for the purpose of histologic diagnosis, it proves to be detrimental to tissue regeneration as cell viability is severely compromised once an extra day is added before tissue recombination and implantation in animals.

We have now changed the protocol to ensure that tissue used for in vivo experiment is processed the same day of surgery or that material is not considered suitable for these expensive and lengthy studies. We have published a paper showing that the model can work to generate robust tumor responses using the oncogene combination Myc and AKT which can serve as a positive control for an aggressive subset of tumors derived from benign human prostate epithelium.

4. Key Research Accomplishments

1) We have established a robust protocol to procure fresh human prostate tissue and isolate subpopulations of prostatic epithelial cells.
2) We have identified the appropriate combinations of cell surface markers for the isolation of NE cells.
3) We have demonstrated that neuroendocrine cells are not required for tumor initiation in a pten conditional knockout mouse prostate cancer model.
4) With the tissue recombination model, tumors can be generated using the neuroendocrine cell-depleted basal cells, suggesting that neuroendocrine cells are not required for tumor initiation in the tissue recombination model.

5. CONCLUSION

Neuroendocrine cells are an important histologic component of benign prostate and prostate cancer. However, because of their rarity, no study has been performed systematically to investigate their function. Through the DOD-sponsored study, we have made important progress in the study of these cells. We have established a robust protocol to procure fresh human prostate tissue and prepare single cell suspensions. We have also demonstrated that with the appropriate combinations of antibodies against cell surface markers, we can reliably isolate different subpopulations of human prostate epithelial cells including neuroendocrine cells. Such cells are viable and can be used to study tumor initiation mechanisms.

We have demonstrated in both a genetically-engineered mouse prostate cancer model and a tissue recombination model that neuroendocrine cells are not required for the initiation of prostate cancer.

An important future direction is to determine if neuroendocrine cells are involved in the progression of prostate cancer to the castration resistant stage. Selection ablation of these cells in an established adenocarcinoma followed by androgen ablation therapy remains an important approach to address this question.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

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65. Tan N, Margolis DJ, Lu DY, King KG, Huang J, Reiter RE, Raman SS. Characteristics of Detected and Missed Prostate Cancer Foci on 3-T Multiparametric MRI Using an Endorectal Coil Correlated With Whole-Mount Thin-Section Histopathology. AJR Am J Roentgenol. 2015 Jul;205(1):W87-92.

Presentations:

Invited by academic institutions:

1. Neuroendocrine Differentiation in Prostate Cancer, UCLA Specialized Program of Research Excellence (SPORE) in Prostate Cancer, April 2011
2. Pathology of Prostate Cancer, Wu Jie-Ping Urologic Center, Beijing University, May 2011
3. Neuroendocrine Differentiation in Prostate Cancer, Anhui Medical University, May 2011
4. Immunohistochemistry in the Differential Diagnosis of Genitourinary Tumors, Anhui Medical University, May 2011
5. Neuroendocrine Differentiation in Prostate Cancer, Department of Urology, Massachusetts General Hospital of Harvard University, June 2011
6. Immunohistochemistry in the Diagnosis and Differential Diagnosis of Genitourinary Tumors. Adicon Clinical Laboratories Inc., Hangzhou, China, November 1, 2011
7. Medical Education in the United States, Anhui Medical University, Hefei, China. November 8, 2011
8. Medical-Legal Issues in the United States, the First Affiliated Hospital of Anhui medical University, Hefei, China, November 10, 2011
9. Tissue recombination technology in cancer research, Cancer Center, Zhejiang University School of Medicine, Hangzhou, July 2012
10. Neuroendocrine differentiation in prostate cancer, Department of Pathology, Yale University School of Medicine, August 2012
11. Neuroendocrine Differentiation in Prostate Cancer, Department of Pathology, University of California at Irvine, Irvine, CA, September 21, 2012
12. Neuroendocrine Differentiation in Prostate Cancer, Department of Urology, Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China, May 2013

13. Neuroendocrine Differentiation in Prostate Cancer, Department of Urology, West China Hospital of Sichuan University, Chengdu, China, June 2013
14. Recent progress in prostate cancer. Wuxi Second People's Hospital, Wuxi, China, November 2013
15. Recent progress in prostate cancer. National Center of Biomedical Analysis (NCBA), Beijing, China, November 2013
16. Therapy-induced aggressive variants of prostate cancer with neuroendocrine differentiation. . Cancer Biology Work-In-Progress Seminar Series, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Beverly Hills, CA, May 2014
17. Pathology of prostate cancer. Pathology Conference, Kaiser Permanente Pathology Department, Los Angeles, CA. May 2014
18. Neuroendocrine differentiation in prostate cancer. Grand Round. Department of Pathology, Loma Linda University School of Medicine. Loma Linda, CA. June 2014
19. Aggressive phenotype of prostate cancer induced by conventional and modern hormonal therapy. Huanxi Hospital of Sichuan University, Chengdu, China, August 2014
20. Aggressive phenotype of prostate cancer induced by conventional and modern hormonal therapy. Eastern China Normal University, Shanghai, China, September 2014
21. Neuroendocrine differentiation in prostate cancer. Grand Round. Los Angeles Biomedical Research Institute May 1, 2015
22. Neuroendocrine differentiation in prostate cancer. Special lecture, Duke University School of medicine, May 4, 2015

Invited by local, national or international conferences:

1. Individualized treatment for cancer. Invited Speaker. Technical Symposium of 49th anniversary of the Chinese-American Engineers and Scientists Association of Southern California (CESASC). San Gabriel, CA, April 2011
2. Tissue recombination technology in the study of tumor initiation cells for prostate cancer. Invited Speaker. Steering Committee Meeting, Intestinal Stem Cell Consortium (ISCC). Stowers Institute, Kansas City, Missouri, May 2011
3. Diagnostic Usage of Immunohistochemistry in Genitourinary Pathology. 4th Biannual MD Anderson Cancer Center-Fudan University Pathology Conference. May 14-15, 2011. Shanghai, China.
4. International session moderator, 18th Annual Meeting of Chinese Urological Association, October 28-30, 2011. Nanjing, China
5. Speaker for Cancer Committee, Zhejiang University-UCLA Joint Center for Research, Hangzhou, China. November 4, 2011
6. Recent Progress in Prostate cancer, Continuing Medical Education Conference of Chinese Urological Association, Huangshan, China, November 12, 2011
7. Panel Member, NCI-MMHCC Pathology Consensus Report Workshop, April 17-18, 2012, New York, NY
8. Pathology of prostate cancer. 2012 Hangzhou International Pathology Symposium, Hangzhou, China, April, 2012
9. Pathology of testicular tumors. 2012 Hangzhou International Pathology Symposium, Hangzhou, China, April, 2012

10. Small cell carcinoma of the prostate: Molecular mechanisms of carcinogenesis. The 7th Forum of Prostate Disease. Shanghai, China, June 2012.
11. International session moderator, 19th Annual Meeting of Chinese Urological Association, November, 2012. Guangzhou, China
12. The annual Hangzhou Symposium on the recent development of molecular diagnosis and treatment of tumors. Hangzhou, China, April 19-21, 2013
13. International forum on translational medicine of urology, Tianjin, China, April 26-28, 2013
14. Neuroendocrine Differentiation in Prostate Cancer, the 2nd Annual PCF China Scientific Symposium, Shanghai, China, May 2013
15. Neuroendocrine Differentiation in Prostate Cancer, Annual Urology Conference, the 2nd Affiliated Hospital of Zhejiang University, Hangzhou, China, May 2013
16. Genitourinary Pathology Case discussion, 2nd Huaxia Pathology Forum, Guilin, China, June 2013
17. Targeted biopsy of prostate cancer, The 7th Annual Forum of Prostate Diseases, Shanghai, China, July 27, 2013
18. Challenges in the Diagnosis of Small Cell Neuroendocrine Carcinoma, The 7th Annual Forum of Prostate Diseases, Shanghai, China, July 27, 2013
19. Pathology and Biology of Neuroendocrine Prostate Cancer, Pathology Workshop for t-NEPC organized by Prostate Cancer Foundation. New York, NY. July 31, 2013
20. Recent progress in prostate cancer. Soochou University Annual Translational Medicine Meeting, Suzhou, China, November 2013
21. Prostate Cancer Stem Cells, Annual Jingmeng Stem Cell Conference, Beijing, China, Feb 15, 2014
22. Aggressive variants of castration-resistant prostate cancer. The Annual Meeting of the Basic Science Division, Chinese Urologic Association, April 26, 2014.
23. Immunohistochemistry in the differential diagnosis of genitourinary tumors. Pathology Symposium organized by Zhejiang Province Quality Control Center, Hangzhou, China, May 2014
24. Pathology of prostate cancer. Pathology Symposium organized by Zhejiang Province Quality Control Center, Hangzhou, China. May 2014.
25. Standardized pathology reporting of prostate biopsy and prostatectomy specimens in the modern era. 8th Annual Forum of Prostate Diseases co-sponsored by Prostate Cancer Foundation and Shanghai Changhai Hospital, September 2014
26. Targeted biopsy of prostate cancer. 8th Annual Forum of Prostate Diseases co-sponsored by Prostate Cancer Foundation and Shanghai Changhai Hospital, September 2014
27. Advancing pathology diagnosis of urologic tumors for better patient care.. International Forum of Urologic Tumors, Shanghai Cancer Hospital of Fudan University, November 2014
28. Therapy-induced changes in metastatic prostate cancer. Jingmeng Stem Cell International Coference, Beijing, China, March 2015
29. Molecular Pathology of Genitourinary Tumors. The Association of Chinese Physicians 20th Annual Convention, Flushing, New York, June 7, 2015

Abstract (Posters and Platform presentations):

1. Song G, Natarajan S, Margolis D, **Huang J**, Dorey F, Macairan ML, Lieu P and Marks LS. Value of Targeted Biopsy in Detecting Prostate Cancer using an Office-Based MR-US Fusion Device. American Urologic Association Annual Meeting, Atlanta, GA, May 2012
2. Gollapudi K, Galet C, Grogan T, Zhang H, **Huang J**, Elashoff D, Gerber L, Freedland S, Rettig M, Aronson W. Is infiltration of tumor-associated macrophages predictive of biochemical recurrence after radical prostatectomy? American Urological Association Annual Meeting, Atlanta, GA May 20, 2012
3. Susan Kerkoutian, Yin Sun, Xinmin Li, Jason Scapa, **Jiaoti Huang**. Cell Type-Specific Biomarkers to Predict the Risk of Prostate Cancer in Men with Increased PSA but Negative Biopsies. United States and Canadian Academy of Pathology Annual Meeting, March 2013, Baltimore, MD
4. Karow D., White N., **Huang J.**, Reiter R., Mattrey R., Margolis D., Raman S., Dale A. Improved Conspicuity and Delineation of High-Grade Prostate Tumors Using "Restriction Spectrum Imaging": Quantitative Comparison with High B-Value ADC. **The International Society for Magnetic Resonance in Medicine** 21st Annual Meeting & Exhibition, 20-26 April 2013, Salt Lake City, Utah, USA
5. Geoffrey Sonn, Shyam Natarajan, Daniel Margolis, Edward Chang, Malu Macairan, Patricia Lieu, **Jiaoti Huang**, Frederick Dorey, Leonard Marks. Prospective Evaluation of MRI-Ultrasound Fusion Biopsy to Diagnose Prostate Cancer in Men with Prior Negative Biopsies. American Urological Society Annual Meeting, May 2013, San Diego, CA
6. Geoffrey Sonn, Shyam Natarajan, Daniel Margolis, Edward Chang, Malu Macairan, Patricia Lieu, **Jiaoti Huang**, Frederick Dorey, Leonard Marks. Targeted Biopsy with MRI-Ultrasound Fusion. American Urological Society Annual Meeting, May 2013, San Diego, CA
7. Nils Kroeger, David B. Seligson, Sabina Signoretti, Hong Yu, Frederic D. Birkhaeuser, Clara Magyar, **Jiaoti Huang**, Joseph Riss, Fairooz F. Kabbinavar, Arie S. Belldegrun, Allan J. Pantuck. Investigating the association of cytoplasmic and nuclear HIF-2 expression with cancer specific survival (CSS) in clear cell renal cell carcinoma. American Society of Clinical Oncology Genitourinary Cancers Symposium, February 2013, Orlando, FL
8. Mitchell Kamrava, Shane Mesko, Robyn Banerjee, **Jiaoti Huang**, D. Jeffrey Demanes, Leonard S. Marks. Quantifying the ki-67 heterogeneity profile in prostate cancer. American Society of Clinical Oncology Genitourinary Cancers Symposium, February 2013, Orlando, FL
9. Lindsay Ann Schelling; Sean Williamson; Jorge Yao; Shaobo Zhang; Mingsheng Wang; **Huang, Jiaoti**; Antonio Lopez; Adeboye Osunkoya; Gregory MacLennan. ERG Immunohistochemistry in Prostatic Small Cell Carcinoma: Comparison to Fluorescence in situ Hybridization. United States and Canadian Academy of Pathology Annual Meeting, March 2013, Baltimore, MD
10. Geoffrey Sonn MD, Edward Chang, Shyam Natarajan, Frederick Dorey, Daniel Margolis , **Jiaoti Huang**, Patricia Lieu, Malu Macairan and Leonard Marks. Impact of MR-Us Fusion Targeted Confirmatory Biopsy on Patient Selection for Active Surveillance. Society for Urologic Oncology Annual Meeting, May 2012, Atlanta, GA

7. INVENTIONS, PATENTS AND LICENSES:

None

8. REPORTABLE OUTCOMES:

None

9. OTHER ACHIEVEMENTS:

Funding applied for based on work supported by this award

1. Cal-Tech-UCLA Joint Center for Translational Medicine Program (PI Huang)
   Period: 6/1/2011 – 5/31/2012
   Title: Biomarkers for prostate cancer
   Direct cost: $50,000
   Role: Principal Investigator

2. Translational Research Funds, UCLA Department of Pathology (PI: Kerkoutian)
   Period: 10/01/11 – 09/31/12
   Title: Predicting the Aggressiveness of Prostate Cancer on Biopsy
   Award Total: $20,000
   Role: Mentor

3. Department of Defense Prostate Cancer Research Program W81XWH-12-1-0206 (PI: L. Wu)
   Period: 07/01/2012 – 06/30/2015
   Title: Disrupting the Pro-Tumorigenic Influences of Tumor-Infiltrating Myeloid Cells by CSF1R Blockade to Augment Androgen Deprivation Therapy in Prostate Cancer
   Direct cost: $150,000/yr
   Percentage effort: 5%
   Role: co-Investigator

4. Stand–up-to-Cancer Dream Team Award (PI: Small and Witte)
   Period: 1/01/2013 -12/31/2015
   Title: Targeting Adaptive Pathways in Metastatic Treatment-Resistant Prostate Cancer
   Direct Cost: $10,000,000 (all 3 years)
   Percentage effort: 10%
   Role: Pathologist

5. Prostate Cancer Foundation Honorable A. David Mazzone Special Challenge Award (PI: Robert Reiter)
   Period: 01/01/2013 – 12/31/2015
   Title: Preventing Treatment Resistance by Co-Targeting Androgen Receptor and SRC/MEK1-Dependent Epithelial to Mesenchymal Transition
   Direct Cost: 2,000,000 (all 3 years)
   Percent effort: 10%
   Role: Co-PI and Pathologist

6. National Cancer Institute 1R01CA172603-01A1 (PI: Jiaoti Huang)

Period: 07/01/2013 – 6/30/2018
Title: A Novel Strategy to Identify Prostate Cancer Biomarkers for Patient Management
Direct cost: $1,037,500 (all 5 years)
Percent effort: 20%
Role: Principle Investigator

7. Jonsson Comprehensive Cancer Center Impact Grant (PI: Sanaz Memarzadeh)
Period: 10/1/14-9/30/15
Title: Restoration of p53 function with a novel structure-based peptide in therapy of solid tumors
Direct cost: $200,000
Percent effort: 3%
Role: co-Principle Investigator

8. UCLA SPORE in Prostate Cancer (2P50CA092131-11A1) (PI: Robert Reiter)
Period: 1/7/2001 – 6/30/2018
Direct cost: $2,300,000/yr
Percentage effort: 10%
Role: Co-Director, Tissue Core

9. National Cancer Institute 1R01CA195505 (Marks, PI)
Period: 5/19/2015 – 4/30/2017
Title: Prospective Assessment of Image Registration for the Diagnosis of Prostate Cancer
Direct cost: $228,750 (annual direct)
Percent effort: 5%
Role: Co-Investigator

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5. Wagner, D. and J. Huang, *Small Cell Carcinoma of the Prostate.* eMedicine from WebMD. , 2009. **http://emedicine.medscape.com/article/1611899-overview**.
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Appendices:

1. Chen H, Sun Y, Wu C, Magyar CE, Li X, Cheng L, Yao JL, Shen S, Osunkoya AO, Liang C, Huang J. Pathogenesis of prostatic small cell carcinoma involves the inactivation of the P53 pathway. Endocr Relat Cancer. 2012 May 24;19(3):321-31.
2. Li Z, Sun Y, Chen X, Squires J, Nowroozizadeh B, Liang C, Huang J. p53 Mutation Directs AURKA Overexpression via miR-25 and FBXW7 in Prostatic Small Cell Neuroendocrine Carcinoma. Mol Cancer Res. 2015 Mar;13(3):584-91.

Endocrine-Related Cancer (2012) **19** 321–331

Pathogenesis of prostatic small cell carcinoma involves the inactivation of the P53 pathway

Hongbing Chen[1,2], Yin Sun[2]*, Chengyu Wu[5], Clara E Magyar[2], Xinmin Li[2,3], Liang Cheng[6], Jorge L Yao[7], Steven Shen[8], Adeboye O Osunkoya[9], Chaozhao Liang[1] and Jiaoti Huang[2,3,4]*

[1]Department of Urology, The Geriatrics Research Institute, First Affiliated Hospital of Anhui Medical University, Anhui, China
[2]Department of Pathology and Laboratory Medicine, [3]Jonsson Comprehensive Cancer Center and [4]Broad Center for Regenerative Medicine and Stem Cell Biology, David Geffen School of Medicine at UCLA, 10833 Le Conte Avenue, 13-229 CHS, Los Angeles, California 90095-1732, USA
[5]Transplant Immunology Laboratory, Albert Einstein College of Medicine, Montefiore Medical Center, Bronx, New York, USA
[6]Department of Pathology, Indiana University School of Medicine, Indianapolis, Indiana, USA
[7]Department of Pathology, University of Rochester School of Medicine, Rochester, New York, USA
[8]Department of Pathology, The Methodist Hospital, Houston, Texas, USA
[9]Department of Pathology, Emory University School of Medicine, Atlanta, Georgia, USA

(Correspondence should be addressed to J Huang at Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA; Email: jiaotihuang@mednet.ucla.edu; C Liang; Email: liang_chaozhao@163.com)

*(H Chen and Y Sun contributed equally to this work)

Abstract

Small cell neuroendocrine carcinoma (SCNC) of the prostate is a variant form of prostate cancer that occurs *de novo* or as a recurrent tumor in patients who received hormonal therapy for prostatic adenocarcinoma. It is composed of pure neuroendocrine (NE) tumor cells, but unlike the scattered NE cells in benign prostate and adenocarcinoma that are quiescent, the NE cells in SCNC are highly proliferative and aggressive, causing death in months. In this study, we provide evidence that interleukin 8 (IL8)–CXCR2–P53 (TP53) signaling pathway keeps the NE cells of benign prostate and adenocarcinoma in a quiescent state normally. While P53 appears to be wild-type in the NE cells of benign prostate and adenocarcinoma, immunohistochemical studies show that the majority of the NE tumor cells in SCNC are positive for nuclear p53, suggesting that the *p53* is mutated. This observation is confirmed by sequencing of genomic DNA showing *p53* mutation in five of seven cases of SCNC. Our results support the hypothesis that *p53* mutation leads to inactivation of the IL8–CXCR2–p53 signaling pathway, resulting in the loss of an important growth inhibitory mechanism and the hyper-proliferation of NE cells in SCNC. Therefore, we have identified potential cells of origin and a molecular target for prostatic SCNC that are very different from those of conventional adenocarcinoma, which explains SCNC's distinct biology and the clinical observation that it does not respond to hormonal therapy targeting androgen receptor signaling, which produces short-term therapeutic effects in nearly all patients with prostatic adenocarcinoma.

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Introduction

Prostate cancer (PC) is the most common malignancy in men and the second leading cause of cancer-related deaths (Cooperberg *et al*. 2004). Normal prostate epithelium contains luminal epithelial cells, basal cells, and a minor component of neuroendocrine (NE) cells that are scattered throughout the prostate (Vashchenko & Abrahamsson 2005, Huang *et al*. 2007, Yuan *et al*. 2007, Sun *et al*. 2009). The majority of PCs are classified as adenocarcinomas characterized by absence of basal cells and uncontrolled proliferation of malignant tumor cells with luminal cell features

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including glandular formation and expression of androgen receptor (AR) and prostate-specific antigen (PSA). Interestingly, every case of prostatic adenocarcinoma also contains a small population (usually ~1%) of NE cells (Vashchenko & Abrahamsson 2005, Huang *et al*. 2007, Yuan *et al*. 2007, Sun *et al*. 2009). The NE cells in adenocarcinoma share many important features with those in the benign prostate. In contrast to the luminal-type non-NE tumor cells, the NE tumor cells do not express AR and PSA (Bonkhoff 2001, Huang *et al*. 2006). Importantly, unlike the bulk, non-NE tumor cells, NE cells in benign prostate and adenocarcinoma are normally quiescent (Bonkhoff 2001, Huang *et al*. 2006).

While adenocarcinomas comprise the majority of PC, there are variant forms among which small cell neuroendocrine carcinoma (SCNC) is an important histological subtype that is often seen in patients with advanced disease. Prostatic SCNC is composed of tumor cells with NE phenotype (Grignon 2004). In comparison to adenocarcinoma, which usually shows glandular formation, SCNC has a solid, sheet-like growth pattern but no glandular formation. Tumor cells are small with fine chromatin pattern, scant cytoplasm, and nuclear molding. Mitotic figures, crush artifact, and tumor necrosis are frequent findings (Yao *et al*. 2006, Huang *et al*. 2007, Wang & Epstein 2008). Although SCNCs may arise *de novo*, such tumors are often seen as recurrent tumors after hormonal therapy for conventional adenocarcinomas of the prostate (Miyoshi *et al*. 2001, Tanaka *et al*. 2001). The prevalence of this disease is likely underestimated because patients with advanced and metastatic diseases usually do not undergo tissue diagnosis. Accurate diagnosis of SCNC is important as such tumors do not respond to hormonal therapy targeting the AR signaling pathway (Brown *et al*. 2003), which produces a short-term therapeutic effect in nearly all cases of prostatic adenocarcinoma (Scher 2003).

The NE tumor cells in prostatic SCNCs share many features with those in benign prostate and prostatic adenocarcinoma. For example, they contain dense-core secretory granules when examined by electron microscopy and are usually positive for NE markers chromogranin A (CgA) and synaptophysin by immunohistochemistry. However, unlike the NE cells in benign prostate and prostatic adenocarcinoma that are quiescent, the NE tumor cells in prostatic SCNCs are highly proliferative, leading to early metastasis (Erasmus *et al*. 2002), and the patients usually die within months of diagnosis. The molecular mechanisms responsible for the aggressive biological behavior of the NE tumor cells in prostatic SCNC remain unknown. Here, we provide evidence that the interleukin 8 (IL8)–CXCR2–p53 (TP53) pathway may be important in keeping the NE cells in benign prostate and prostatic adenocarcinoma in a quiescent state, and inactivation of the pathway due to *p53* mutation may be responsible for the rapid proliferation of NE cells in prostatic SCNC.

Materials and methods

Cell culture and transfection

LNCaP cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 maintained at 37 °C. pcDNA3 vector or pcDNA3-CXCR2 was transfected into LNCAP cells with Lipofectamine 2000 (Invitrogen), and stable clones were selected with G418 at 300 μg/ml for 30 days. For siRNA transfection, Dharmafect #2 was used with siRNA at 100 nM for *p53* (duplex sequences are 5′-rCrCrA rCrCrArUrCrCrArCrUrArCrArArCr-UrArCrArUrGTG-3′, 5′-rCrArCrArUrGrUrArGrUr-UrGrUrArGrUrGrGrArUrGrGrUrGrGrUrA-3′). For expression of *p53* in PC3 cells, GFP expression plasmid with a ratio of 9:1 to CMV-driven *p53* plasmid were transfected with Fugene 6, 3 days later, IL8 was added, and the number of GFP-positive cells were counted using Acumen laser cytometer.

Immunoblot analysis

Cells were washed with PBS and lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitor cocktail for 15 min at 4 °C. Cell lysates were centrifuged and supernatants were collected. Equal amounts of proteins were resolved on SDS–PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. The resulting blots were blocked in 5% nonfat dry milk for 30 min followed by incubation with primary antibody against CXCR2 (Cat. no. 555932; BD Biosciences, San Diego, CA, USA). HRP-conjugated secondary antibody and supersignal west pico chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA) were used to visualize antigen–antibody complexes.

Flow cytometry analysis

Flow cytometry analysis was performed as described previously (Palapattu *et al*. 2009) using a fluorescein

isothiocyanate (FITC) conjugated anti-CXCR2 antibody (BD Biosciences).

Cell proliferation assay

Cells were seeded into 96-well microplates and 10 nM IL8 were added to the culture 24 h after seeding for 6 days with the cell number determination at days 0, 2, 4, and 6 using CellTiter Glo assay (Promega) according to the manufacture's protocol.

RNA isolation and quality control and microarray hybridization

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and purity/concentration was determined using a NanoDrop 8000 (NanoDrop Products, Wilmington, DE, USA). Microarray targets were prepared using MessageAmp Premier RNA Amplification Kit (Ambion) and hybridized to the Affymetrix GeneChip U133plus 2.0 Array (Affymetrix, Inc., Santa Clara, CA, USA) according to the manufacturers' instructions. The arrays were washed and stained with streptavidin phycoerythrin in Affymetrix Fluidics Station 450 using the Affymetrix GeneChip protocol and then scanned using an Affymetrix GeneChip Scanner 3000.

Microarray data analysis

The acquisition and initial quantification of array images were conducted using the AGCC Software (Affymetrix, Inc.). The subsequent data analyses were performed using Partek Genomics Suite version 6.4 (Partek, Inc., St. Louis, MO, USA). Differentially expressed genes were selected at ≥ 1.5-fold and $P < 0.05$. Cluster analyses and principal component analysis were conducted with Partek default settings. Biofunctional analysis was performed using Ingenuity Pathways Analysis Software version 7.6 (Ingenuity Systems, Redwood City, CA, USA).

Construction of tissue microarrays and immunohistochemical staining

Construction of tissue microarray (TMA) and immunohistochemical staining has been described previously (Huang *et al*. 2005). Three hundred cases of prostatectomy specimens were reviewed and representative cancer and benign areas were circled. The cancer and benign prostate tissues were then transferred from donor blocks to recipient blocks to construct tissue microarray blocks. H&E sections were prepared from the top for quality control.

For immunohistochemical studies, paraffin sections of 5 μm thickness were prepared from regular histological blocks or from tissue microarrays. The sections were deparaffinized with xylene and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Heat-induced antigen retrieval (HIER) was carried out for all sections in 0.01 M citrate, pH = 6.00, using a vegetable steamer at 95 °C for 25 min. Mouse monoclonal anti-p53 antibody (clone 1801, Oncogene, OP09-100 μg, used at 1:50) and mouse monoclonal anti-CgA antibody (clone DAK-A3, M0869, used at 1:1000; DakoCytomation Carpinteria, CA, USA) were used as primary antibodies. MACH2 Mouse HRP-Polymer (Cat no. #MHRP520L, Biocare Medical, Concord, CA, USA) was used as the secondary antibody. For double staining of p53 and CgA, the two antibodies were used sequentially. 3,3'-diaminobenzidine (DAB, brown) and alkaline phosphatase (AP, red) were used as chromogens.

Genomic sequencing of p53 exons

Based on microscopic examination, small cell carcinoma regions in paraffin section were scraped off and collected from the slides followed by deparaffinization and overnight proteinase K digestion. The genomic DNA was purified through ethanol precipitation after phenol and chloroform extraction. Exons 5–10 of *p53* were amplified by PCR followed by gel electrophoresis analysis. The PCR products were either directly used for sequencing if there is specific amplification or gel purified and sequenced by one of the amplifying primers. Primers for amplifications are as follows: exon 5 forward 5′-GCCCTGTCGTCTCTCCAG-3′, reverse 5′-GACTTTCAACTCTGTCTCCTTCC-3′; exon 6 forward 5′-CTTAACCCCTCCTCCCAGAG-3′, reverse 5′-CAGGCCTCTGATTCCTCACT-3′; exon 7 forward 5′-GTGTGCAGGGTGGCAAGT-3′, reverse 5′-CGACAGAGCGAGATTCCATC-3′; exons 8 and 9 forward 5′-CGGCATTTTGAGTGTTAGACTG-3′, reverse 5′-GCCTCTTGCTTCTCTTTTCC-3′, exon 10 forward 5′-TGCATGTTGCTTTTGTACCG-3′, reverse 5′-GGAGTAGGGCCAGGAAGG-3′.

Results

Establishing LNCaP cell lines stably expressing IL8 receptor CXCR2

We have previously shown that NE cells in benign prostate and prostatic adenocarcinoma express IL8 and its receptor CXCR2 (Huang *et al*. 2005). We hypothesized that the autocrine action of IL8–CXCR2

may be responsible for certain function of NE cells (Huang *et al*. 2005), but the exact consequence of this autocrine action was unclear. A more recent publication showed that activation of CXCR2 by IL8 leads to cellular senescence, a state of stable proliferative arrest, via a p53-dependent mechanism (Acosta *et al*. 2008). Thus, we further hypothesized that IL8–CXCR2–p53 pathway may also be active in NE cells in benign prostate and prostatic adenocarcinoma, which maintains these cells in a quiescent state.

In human PC tissue, NE cells are positive for IL8 and its receptor CXCR2 while luminal cells are negative for both (Huang *et al*. 2005). LNCaP cells have features of prostatic cancer cells with luminal (non-NE) features in that they express luminal differentiation markers AR and PSA but not NE markers CgA and neuron-specific enolase (NSE) (Palapattu *et al*. 2009). Accordingly, there is no detectable expression of IL8 and a very low level of CXCR2 in LNCaP cells.

In order to study the function and signaling pathway of CXCR2, we transfected LNCaP cells with pcDNA3-CXCR2 plasmid to obtain LNCaP cells that stably overexpress CXCR2. Figure 1 (A, B and C) shows that we have established LNCaP cells that overexpress CXCR2 (LNCaP/CXCR2 cells), as confirmed by immunoblot as well as flow cytometric analysis with antibodies against CXCR2.

Activation of CXCR2 by IL8 inhibits cell proliferation

In a genetic screen to identify molecules that are critical for replicative senescence, it was found that activation of CXCR2 by IL8 leads to cellular senescence, which is mediated by p53 (Acosta *et al*. 2008). Thus, we hypothesized that in NE cells of benign prostate and adenocarcinoma, the IL8–CXCR2–p53 pathway may be responsible for keeping the cells in a quiescent state normally. To test this hypothesis, we used LNCaP and LNCaP/CXCR2 cells and examined their proliferative responses to IL8. As shown in Fig. 1D, IL8 inhibited the proliferation of LNCaP/CXCR2 cells but not the parental LNCaP cells, a finding that supports our hypothesis. We also examined the transcriptional responses to IL8 by comparing the transcriptional profiles of cells with or without IL8 treatment. As shown in Fig. 1E, IL8 stimulation induced similar changes in transcriptional profiles at 1 and 12 h, with up- or downregulation of a variety of genes. Interestingly, activation of CXCR2 by IL8 led to altered expression of many genes involved in p53 signaling. Functional gene family analysis showed that among the genes whose expression increased significantly in response to IL8 stimulation were those controlling the G1/S and G2/M transition check points. As an important function of p53 is in the cell cycle control and growth inhibition, these results suggest that growth inhibition of LNCaP/CXCR2 cells after activation of CXCR2 by IL8 may involve the p53 pathway.

P53 is required for the growth inhibitory function of the IL8–CXCR2 autocrine pathway

P53 was shown to be critical in mediating the IL8–CXCR2 signaling to induce cellular senescence (Acosta *et al*. 2008). Similarly, p53 may also be essential in the growth suppression after activation of CXCR2 by IL8 treatment in LNCaP/CXCR2 cells, as suggested by our microarray studies. To directly test this hypothesis, we attempted to abrogate p53 function in LNCaP/CXCR2 cells to determine whether a loss of P53 activity would change the growth response toward IL8 treatment. P53 protein is of wild-type in LNCaP cells (van Bokhoven *et al*. 2003), thus inactivation of p53 activity can be achieved through reduction of the endogenous wild-type protein. We used a transient transfection method to introduce siRNA to reduce the endogenous P53 level in LNCaP/CXCR2 cells. As shown in Fig. 2A, we were able to significantly reduce the P53 level by siRNA method. Importantly, IL8 treatment of the cells with reduced P53 levels resulted in an increase in cell number while the control cells showed reduced cell numbers in response to IL8 treatment, suggesting that P53 is critically important for the growth inhibition after activation of CXCR2 by IL8. The results also suggest that in the absence of functional P53, activation of CXCR2 by IL8 may stimulate cell proliferation via a different signaling pathway.

Our group has recently demonstrated that another commonly used PC cell line, PC3, possesses features of prostatic SCNC in that these cells are negative for luminal differentiation markers AR and PSA but positive for NE markers (Tai *et al*. 2011). Similar to NE cells in benign prostate and prostatic adenocarcinoma, PC3 cells express IL8 (Ma *et al*. 2009) and CXCR2 (Reiland *et al*. 1999). Contrary to NE cells in benign prostate and prostatic adenocarcinoma, PC3 cells are highly proliferative NE cells similar to human prostatic SCNC. We hypothesized that loss of the growth inhibitory function of the IL8–CXCR2–p53 pathway may be responsible for the rapid proliferation and aggressive behavior of PC3 cells. In support of our hypothesis, it has been reported that PC3 cells, unlike LNCaP cells, contain a *p53* mutation

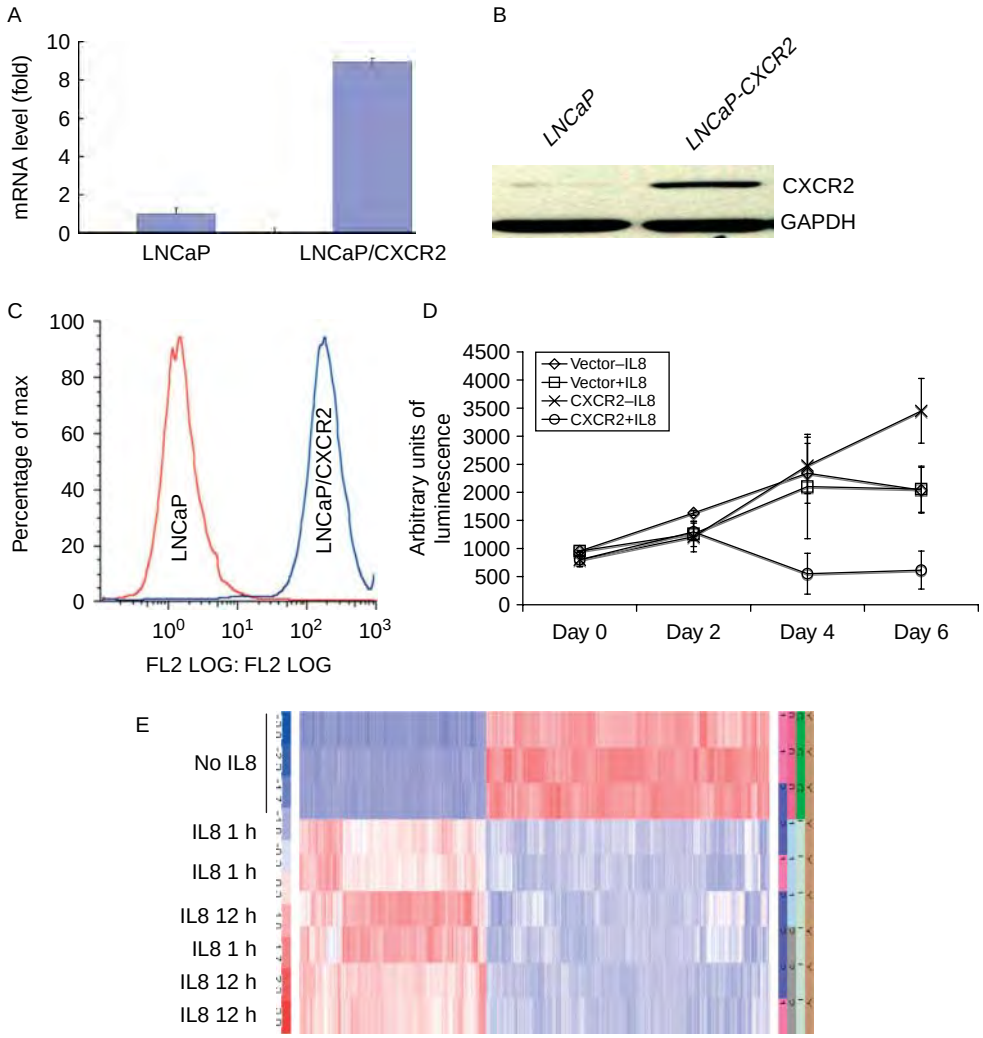

Figure 1 Activation of CXCR2 by IL8 inhibits cell proliferation and induces changes in gene expression profile. LNCaP cells were stably transfected with a plasmid-expressing CXCR2, a receptor for IL8. Real-time PCR (A), western blot (B), and flow cytometry (C) analyses show that CXCR2 is not expressed in the parental LNCaP cells but highly overexpressed in the stable cell line. (D) LNCaP cells transfected with vector control or CXCR2 were cultured in the absence or presence of IL8. IL8 significantly inhibits the proliferation of LNCaP cells expressing CXCR2. (E) Microarray analysis shows that IL8 stimulation of LNCaP/CXCR2 cells results in significant changes of gene expression profile. Similar changes were observed at 1 and 12 h after IL8 treatment.

(van Bokhoven *et al*. 2003). Therefore, we tested whether forced expression of wild-type P53 may restore the function of the IL8–CXCR2–P53 pathway resulting in inhibition of cellular proliferation. Similar to what was reported previously (Isaacs *et al*. 1991), introduction of wild-type P53 significantly slowed the growth of PC3 cells. Additionally, in contrast to control PC3 cells, IL8 treatment of PC3 cells expressing wild-type P53 led to reduced cell numbers. These data suggest that IL8–CXCR2–p53 pathway plays a central role in mediating growth suppression normally, and loss of function of this pathway (such as a P53 mutation) can lead to increased proliferation in NE cells.

Mutation of *P53* in NE tumor cells of prostatic SCNC

NE cells in benign human prostate and prostatic adenocarcinoma are quiescent (Huang *et al*. 2007) but those in prostatic SCNC, whether arising *de novo* or as a recurrent tumor in patients treated with hormonal therapy for adenocarcinoma, are highly proliferative and biologically very aggressive (Yao *et al*. 2006, Wang & Epstein 2008). The underlying molecular mechanism responsible for such a difference is unclear. The results presented above are consistent with the model that the intact IL8–CXCR2–P53 pathway may be responsible for keeping the NE cells

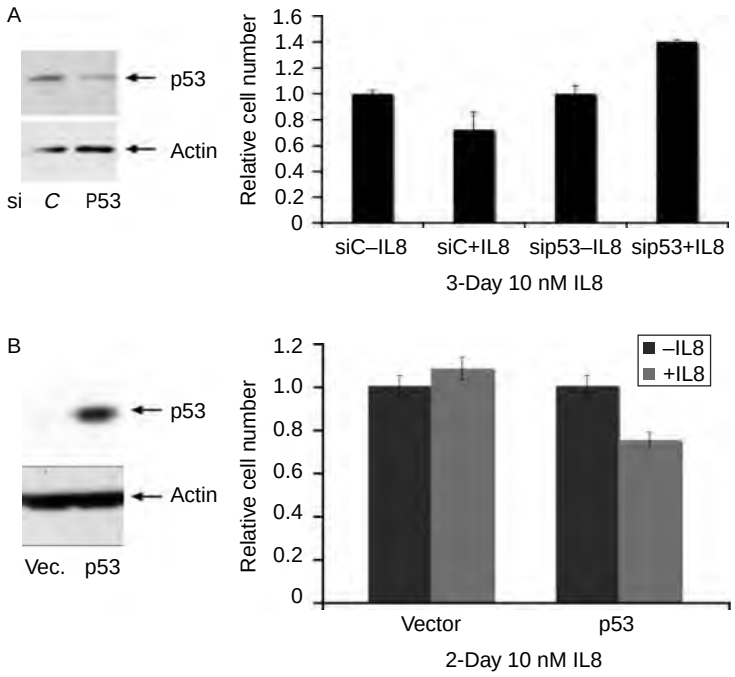

Figure 2 p53 is important for mediating IL8 activity in LNCAP and PC3 cells. (A) Knocking down endogenous p53 in CXCR2-expressing LNCaP cells leads to increase in cell proliferation in response to IL8 treatment. (B) Expression of p53 renders PC3 cells responsive to growth inhibition of IL8. GFP-positive cells were counted using laser microplate cytometer as equivalent to p53 expression in PC3 cells. The ratio of the number of cells in the presence or absence of IL8 was tabulated.

under a state of quiescence, a mechanism that has been reported in senescent cells (Acosta *et al*. 2008). Therefore, we further hypothesized that the reason the NE tumor cells in prostatic SCNC are highly proliferative and biologically aggressive is because this important growth-inhibitory pathway is inactivated in such cells. As mutation of *P53* represents the most common genetic alteration of human malignancies, we further hypothesized that *P53* mutation is a fundamental molecular change responsible for the aggressive biological behavior of prostatic SCNC as has been observed in PC3 cells, a cell line that is characteristic of human prostatic SCNC (Tai *et al*. 2011).

It is well known that *p53* mutation causes prolonged half-life and subsequent nuclear accumulation of the p53 protein, which then becomes detectable by immunohistochemistry (Schlomm *et al*. 2008, Kudahetti *et al*. 2009). Therefore, we performed immunohistochemistry to observe p53 nuclear staining in NE cells of benign prostate, prostatic adenocarcinoma, and SCNC. Adjacent sections were prepared from tissue microarrays containing 150 cases (300 cores) of prostatic adenocarcinoma and an equal number of benign prostate from the same patients. We had shown that the two adjacent sections prepared in this manner contained essentially identical cells and such sections are useful in identifying proteins specifically expressed in prostatic NE cells (Huang *et al*. 2005, 2006, Wu *et al*. 2006). To determine whether NE cells in benign prostate tissue and adenocarcinoma are positive for nuclear p53 staining, the first sections from the tissue microarrays were stained with an anti-CgA antibody to highlight NE cells as CgA is widely considered the most sensitive and specific marker for prostatic NE cells (Vashchenko & Abrahamsson 2005, Huang *et al*. 2007, Yuan *et al*. 2007) and the second sections stained with an anti-p53 antibody. The stained slides were scanned and analyzed using the Ariol SL-50 scanner with thresholds applied for RGB algorithms, shapes, and sizes. The number of nuclei and positively stained cells were counted and separately recorded. Benign cores ($n=300$) in the TMAs contained 1353 NE cells (representing 0.33% of total cells) and 3297 p53+ cells (representing 0.84% of total nuclei); cancer cores ($n=300$) had 1331 NE cells (representing 0.23% of total cells) and 4646 p53+ cells (representing 0.9% of total nuclei). As has been described previously, the NE cells were present as individual cells or small clusters (Huang *et al*. 2007). The p53-positive cells in benign prostate appear to be mostly basal cells while in adenocarcinoma, the p53-positive cells were

mostly individual tumor cells. Importantly, when the corresponding cores in the two TMA sections were matched and compared, there was no overlap between CgA+ and p53+ cells, supporting the conclusion that the NE cells were negative for p53 nuclear staining and the scattered nuclear p53+ cells were not the NE cells (Fig. 3A and B). To confirm this finding, we also performed double staining for CgA and p53 on the same TMA section. We again showed that CgA staining (cytoplasmic) and p53 staining (nuclear) did not exist in the same cells (Fig. 3C). These results suggest that in benign prostate and prostatic adenocarcinoma, the NE cells likely have wild-type p53.

To determine whether *p53* mutation may be present in prostatic SCNC, we used immunohistochemistry to study *p53* expression in 31 cases of prostatic SCNC using regular histological sections. Unlike NE cells in benign prostate or prostatic adenocarcinoma, the NE tumor cells in prostatic SCNCs were positive for p53, with 74% (23/31) of the cases showing positivity in 50–100% cells and the remaining 26% (8/31) of the cases with positivity in 5–50% cells (Fig. 4A and B). As nuclear staining of p53 results from prolonged half-life of the protein after mutations, these results suggest that most, if not all, prostatic SCNCs contain mutations in the *p53* gene.

To directly examine the possible mutations of the *p53* gene in the SCNC, we obtained formalin-fixed, paraffin-embedded tissue from seven cases of prostatic SCNC and extracted genomic DNA from them. Exons 5–10 of *p53* were amplified by PCR followed by direct sequencing. As shown in Fig. 4C, this analysis indicated that five out of seven tumor samples contain an identical allele of a missense transition converting G to A at position 747, changing negatively charged aspartic acid to hydrophilic amino acid asparagine at amino acid 184, again supporting our hypothesis that

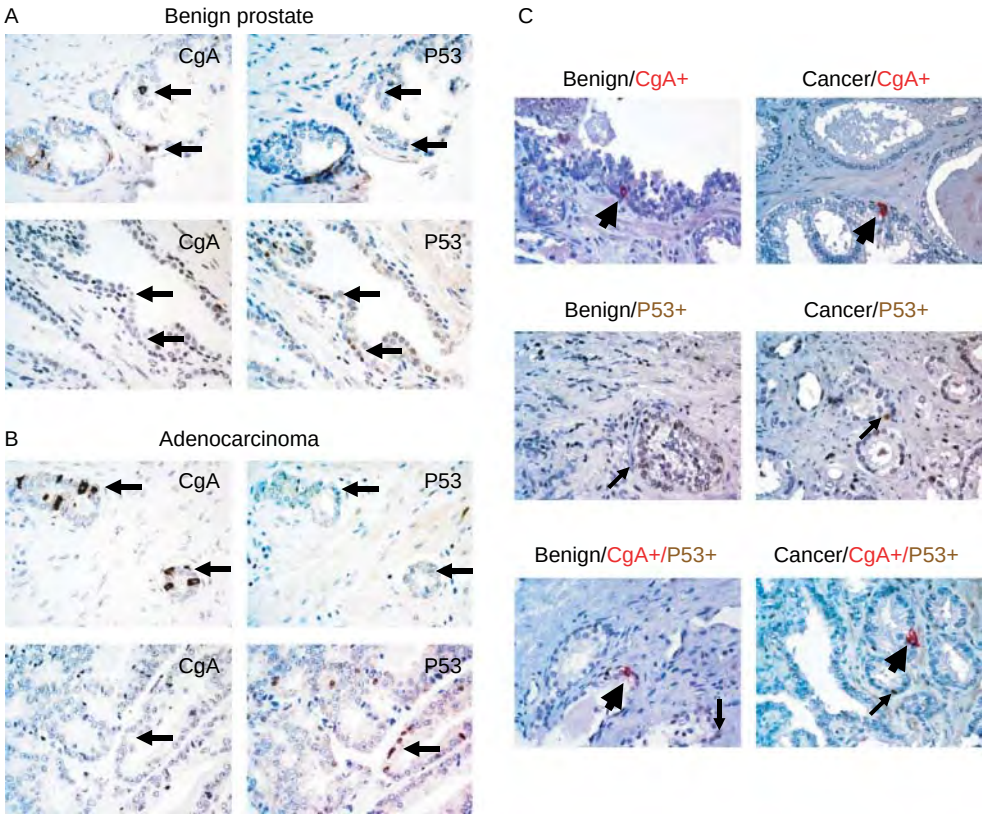

Figure 3 NE cells in benign and adenocarcinoma of prostate are negative for nuclear P53 staining. (A) Adjacent sections of benign prostate were stained with anti-CgA and anti-P53 antibodies respectively. The upper panel shows that NE cells (CgA+, arrows) are negative for nuclear P53. The lower panel shows that the scattered P53+ cells (arrows) were not NE cells (CgA−). (B) Adjacent sections of prostate adenocarcinoma were stained with anti-CgA and anti-P53 antibodies respectively. The upper panel shows that NE cells (CgA+, arrows) are negative for nuclear P53. The lower panel shows that the scattered P53+ cells (arrows) were not NE cells (CgA−). (C) Sections of benign prostate and prostate adenocarcinoma were doubly stained for CgA (cytoplasmic, red, thick arrow) and P53 (nuclear, brown, thin arrow). The pictures on the left show benign prostate with NE cells (thick arrow, top), P53+ cells (thin arrow, middle), and both (lower). The right panel shows the same in adenocarcinoma. Importantly, cytoplasmic CgA staining and nuclear P53 staining do not occur in the same cells.

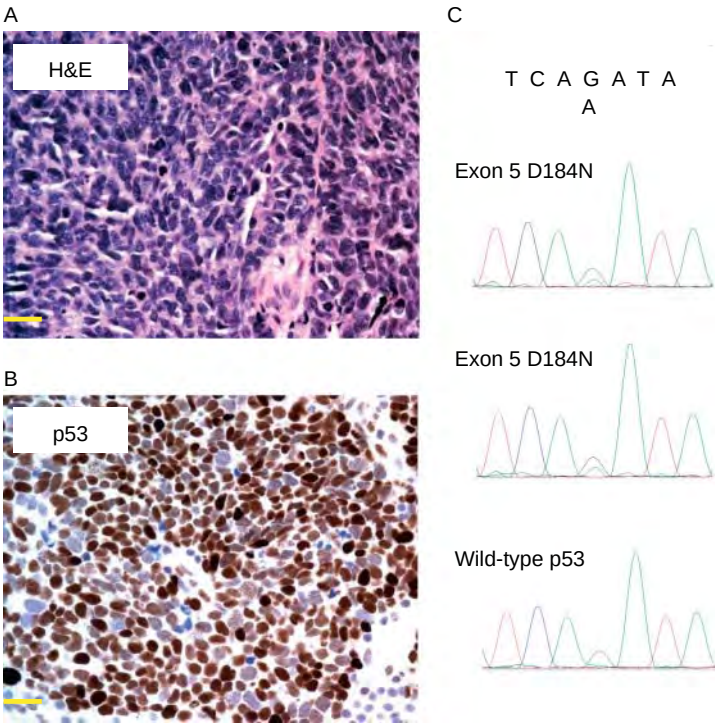

Figure 4 SCNC shows diffuse nuclear P53 staining and contains P53 mutation. (A) H&E slide of SCNC composed of pure NE tumor cells with characteristic morphological features including scant cytoplasm, fine nuclear chromatin pattern, and nuclear molding. (B) Immunohistochemistry shows diffuse nuclear P53 staining in SCNC. (C) Sequencing data from SCNC. The two examples in the top and middle panels show p53 mutation in exon 5 resulting in replacement of aspartic acid at position 184 by asparagine. The bottom panel shows wild-type P53 sequence in the same region.

a *p53* mutation inactivates the IL8–CXCR2–p53 pathway that normally keeps the NE cells in a quiescent state, resulting in hyper-proliferation and aggressive behavior, features that are characteristic of the tumor cells in SCNC.

Discussion

NE cells are normally present as a minor component in benign prostate and prostatic adenocarcinomas and their functions remain unclear. It has been observed that in cultured LNCaP cells, a cell line with luminal cell features, androgen withdrawal induces differentiation toward a NE phenotype (Burchardt *et al*. 1999), suggesting that hormonal therapy in PC patients may drive luminal-type cancer cells into NE cancer cells. One interesting hypothesis is that the normally quiescent NE tumor cells may provide growth signals to the adjacent non-NE cancer cells through a paracrine mechanism, contributing to therapeutic failure and the progression to castration-resistant state (Vashchenko & Abrahamsson 2005, Huang *et al*. 2007, Yuan *et al*. 2007, Sun *et al*. 2009). Occasionally, the recurrent tumor contains pure populations of highly aggressive NE tumor cells and is classified as SCNC, although SCNC can also be seen in patients without a history of adenocarcinoma. With the advent of novel drugs such as Abiraterone and MDV3100 that show superior efficacy in the inhibition of AR signaling, we expect that the incidence of prostatic SCNC will only increase.

The cell of origin and the molecular basis for prostatic adenocarcinoma remain controversial. These issues are even less clear for prostatic SCNC. Our results are consistent with a model in which the NE cells in benign prostate and prostatic adenocarcinoma are normally quiescent due to the growth inhibitory function of an autocrine mechanism involving the IL8–CXCR2–p53 pathway. Mutation of *p53* leads to inactivation of the above pathway leading to hyper-proliferation of NE cells with the resultant SCNC as shown in Fig. 5. In fact, in the absence of functional P53, activation of CXCR2 by IL8 appears to stimulate cell growth (Fig. 2), suggesting that CXCR2 may elicit both growth inhibitory and growth stimulatory pathways. Normally, the P53-mediated growth-inhibitory pathway may dominate. Once P53 is mutated, CXCR2 activation becomes growth promoting and oncogenic. This hypothesis is supported by previous reports showing

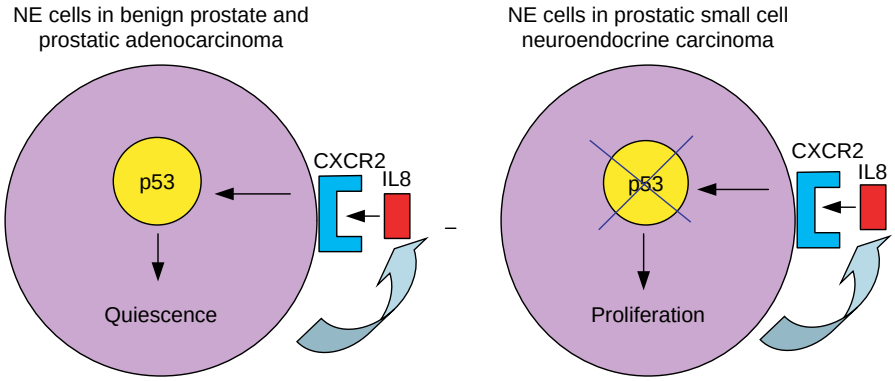

Figure 5 The function of IL8–CXCR2–p53 pathway in controlling the proliferation of NE cells in benign prostate, adenocarcinoma, and SCNC. The model on the left suggests that autocrine activation of CXCR2 by IL8 activates the p53 pathway, which keeps NE cells of benign prostate and prostate adrenocarcinoma in a quiescent state. The model on the right suggests that p53 mutation inactivates the IL8–CXCR2–p53 pathway, leading to rapid proliferation and aggressive biological behavior of NE tumor cells in SCNC.

that a CXCR2-neutralizing antibody can inhibit PC3 cell proliferation (Reiland *et al.* 1999) and deletion of CXCR2 inhibits TRAMP tumors (Shen *et al.* 2006).

Therefore, we have identified a potential cell-of-origin as well as a molecular target for prostatic SCNS. It is unclear whether the NE cells in both benign prostate and adenocarcinoma can be the cells of origin for SCNS. It is possible that pure SCNC arising *de novo* may result from *p53* mutation in NE cells of benign prostate, although we cannot exclude the possibility that SCNC always arises from NE cells of adenocarcinoma, but in some cases, the rapidly proliferating tumor NE cells have completely overtaken the slow-growing adenocarcinoma, resulting in the histological appearance of a pure SCNC. For those SCNCs that coexist with prostatic adenocarcinoma and those that are recurrent tumors in patients with a history of adenocarcinoma treated with hormonal therapy, the most likely mechanism appears to be *p53* mutation in the NE cells of adenocarcinoma. For these patients, a likely scenario is that within the adenocarcinoma, a *p53* mutation occurs in NE cell(s) during the course of hormonal therapy leading to the development of SCNC. The rapidly proliferating NE cells of SCNC can coexist with adenocarcinoma initially but eventually become the only component as adenocarcinoma cells usually have a low proliferation rate and this component can be completely overtaken by SCNC. Because of its distinct cell of origin and molecular alteration, SCNC is an entirely different disease from adenocarcinoma. In contrast to adenocarcinoma, prostatic SCNC is a disease of NE differentiation, likely has its cell of origin in NE cells of benign prostate and/or adenocarcinoma, with *p53* mutation as the underlying molecular mechanism, and does not respond to hormonal therapy, which has proven to be effective, at least initially, in nearly all patients with prostatic adenocarcinomas.

Our results are consistent with findings in cell line models. We have shown that PC3 cells, a PC cell line originally derived from a patient's bone metastasis, have NE features in that they express NE markers CgA and NSE (Palapattu *et al.* 2009). Like NE cells in human prostate and prostatic adenocarcinoma, PC3 cells express IL8 (Ma *et al.* 2009) and IL8 receptor CXCR2 (Reiland *et al.* 1999) and are positive for CD44 (Palapattu *et al.* 2009). Unlike NE cells in benign prostate and adenocarcinoma, PC3 cells are highly proliferative and biologically aggressive, features shared by SCNCs. Consistent with this notion, it has been shown that PC3 cells contain a *p53* mutation and restoration of p53 function inhibits tumor cell growth (Isaacs *et al.* 1991). Therefore, we have proposed that PC3 cells may actually represent a cell line of SCNC (Tai *et al.* 2011).

Our model is supported by animal models of PC. Transgenic mice expressing SV40 early genes including T antigen (TRAMP) develop aggressive carcinomas with abundant NE tumor cells similar to human prostatic SCNC (Greenberg *et al.* 1995, Masumori *et al.* 2001, 2004), as do $p53^{-/-}Rb^{-/-}$ double knockout mice (Zhou *et al.* 2006). In both models, the AR-responsive probasin promoter was used to drive the expression of the transgene or the Cre recombinase. Although AR is not usually expressed in NE cells, it is possible that a low level of AR is present in NE cells at some point of mouse prostate development and is sufficient to activate the probasin promoter. We hypothesize that in these animals, inactivation of p53 in NE cells of the prostate leads to malignant transformation and rapid proliferation of

NE cells, resulting in the development of a malignant tumor with abundant NE tumor cells mimicking human prostatic SCNC. Notably, in both models, there are also areas of glandular formation resembling adenocarcinoma, which probably results from inactivation of p53 and/or Rb in luminal epithelial cells. In most of the TRAMP tumor tissues we have examined, NE tumor cells predominate, likely reflecting the highly proliferative nature of the malignant NE tumor cells.

The findings described here are also consistent with a previous report by De Marzo's group who reported positive nuclear staining for p53 and a *p53* mutation in a case of SCNC intermixed with adenocarcinoma (Hansel *et al*. 2009). The *p53* mutation discovered in their study differs from that identified in our cases, suggesting that a variety of *p53* mutations may be found if a large number of SCNCs are sequenced.

It is noteworthy that SV40 T antigen inactivates both p53 and Rb in TRAMP tumors and the Nikitin group has shown that both p53 and Rb need to be inactivated in order for invasive tumors to develop (Zhou *et al*. 2006). It is possible that, inactivation of Rb as well as p53 in NE cells is required for the development of prostatic SCNC in men. However, unlike p53 for which mutations are usually associated with protein accumulation in the nucleus detectable by immunohistochemistry, assaying for the inactivation of Rb pathway is not straightforward. Therefore, additional studies need to be performed to determine whether the Rb pathway, which appears to be important for prostatic adenocarcinoma (Balk & Knudsen 2008), is also involved in the pathogenesis of prostatic SCNC in men.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H Chen, Y Sun, L Cheng, C Wu, J L Yao, S Shen, A O Osunkoya, C Liang, and J Huang designed the study; H Chen, Y Sun, and C Wu performed the experiments; Y Sun, C E Magyar, X Li, and J Huang analyzed the data; J Huang wrote the manuscript.

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Signal Transduction

Molecular Cancer Research

p53 Mutation Directs AURKA Overexpression via *miR-25* and FBXW7 in Prostatic Small Cell Neuroendocrine Carcinoma

Zhen Li[1], Yin Sun[1], Xufeng Chen[1], Jill Squires[1], Behdokht Nowroozizadeh[1], Chaozhao Liang[2], and Jiaoti Huang[1]

Abstract

Prostatic small cell neuroendocrine carcinoma (SCNC) is a rare but aggressive form of prostate cancer that is negative for androgen receptor (AR) and not responsive to hormonal therapy. The molecular etiology of this prostate cancer variant is not well understood; however, mutation of the p53 (*TP53*) tumor suppressor in prostate neuroendocrine cells inactivates the IL8–CXCR2–p53 pathway that normally inhibits cellular proliferation, leading to the development of SCNC. SCNC also overexpresses Aurora kinase A (AURKA) which is considered to be a viable therapeutic target. Therefore, the relationship of these two molecular events was studied, and we show that p53 mutation leads to increased expression of *miR-25* and down-regulation of the E3 ubiquitin ligase FBXW7, resulting in elevated levels of Aurora kinase A. This study demonstrates an intracellular pathway by which p53 mutation leads to Aurora kinase A expression, which is critically important for the rapid proliferation and aggressive behavior of prostatic SCNC.

Implications: The pathogenesis of prostatic SCNC involves a p53 and Aurora Kinase A signaling mechanism, both potentially targetable pathways. *Mol Cancer Res; 1–8. ©2014 AACR.*

Introduction

Prostate cancer is the leading cause of cancer-related death for men in western countries. Understanding the molecular mechanisms of prostate carcinogenesis and progression is the foundation and a challenge for the development of effective therapy. Patients with low grade and early stage of prostate cancers can be cured by surgery or radiotherapy. For those with advanced and metastatic prostate cancers that are not amenable for local therapies, hormonal therapy targeting androgen receptor (AR) pathway has been the treatment of choice for many decades. Unfortunately, this therapy is not curative, and the cancer invariably progresses to castration-resistant state with few therapeutic options.

The majority of human prostate cancers are classified as adenocarcinoma with the bulk tumor cells showing luminal differentiation including the expression of AR and PSA. Interestingly, all adenocarcinomas of the prostate contain some neuroendocrine (NE) cells (1, 2). Unlike the bulk tumor cells, the scattered NE tumor cells are usually quiescent. In contrast, an important histologic variant prostate cancer called small cell neuroendocrine carcinoma (SCNC) is composed of NE tumor cells that are highly proliferative and aggressive. Although SCNC is occasionally diagnosed in patients without any previous history of prostate cancer, it more commonly occurs as a recurrent tumor in patients with a history of adenocarcinoma who have received hormonal therapy. It has been suggested that the novel drugs abiraterone and enzalutamide (formerly known as MDV3100) that further inhibit AR signaling will induce even more cases of SCNC.

We recently demonstrated that the IL8/CXCR2/p53 signaling pathway keeps the NE cells in adenocarcinoma in a quiescent state, and mutant p53 inactivates this pathway, leading to hyperproliferation of NE cells and the development of SCNC (3). Meanwhile, previous study also found that Aurora kinase A was overexpressed in the majority of cases of SCNC, indicating a potential role of Aurora Kinase A in the development of SCNC (4).

In this study, we provide evidence showing that p53 mutation leads to elevated expression of Aurora kinase A through regulation of *miR-25* and FBXW7 (F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase) thus revealing a potential molecular mechanism of p53 mutation in promoting the rapid proliferation and aggressive behavior of NE tumor cells in prostatic SCNC.

Materials and Methods

Cell lines

Human prostate LNCaP Clone FGC, PC-3, and NCI-H660 cells were from American Type Culture Collection (ATCC) and were authenticated utilizing short tandem repeat profiling. LNCaP Clone FGC cells were cultured in ATCC-formulated RPMI-1640

[1]Department of Pathology and Urology, Jonsson Comprehensive Cancer Center and Broad Center of Regenerative Medicine and Stem Cell Research, UCLA David Geffen School of Medicine, Los Angeles, California. [2]Department of Urology, The First Affiliated Hospital of Anhui Medical University, Hefei, China.

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Z. Li and Y. Sun contributed equally to this article.

Current address for Y. Sun: Department of Radiation Oncology, University of Rochester Medical Center School of Medicine and Dentistry, Rochester, NY.

Corresponding Authors: Jiaoti Huang, UCLA David Geffen School of Medicine, 10833 Le Conte Ave., 13-226 CHS, Los Angeles, CA 90095. Phone: 310-267-2264; Fax 310-794-4161; E-mail: JiaotiHuang@mednet.ucla.edu; or Yin Sun, Yin_Sun@URMC.Rochester.edu

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medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 maintained at 37°C. PC-3 cells were cultured in ATCC-formulated F-12K medium with 10% FBS. NCI-H660 cells were cultured in RPMI-1640 medium with 0.005 mg/mL insulin, 0.01 mg/mL transferrin, 30 nmol/L sodium selenite, 10 nmol/L hydrocortisone, 10 nmol/L beta-estradiol, 4 mmol/L L-glutamine, and 5% FBS (HITES medium). NE1.8 cells were provided by Dr. Ming-Fong Lin (5), and were cultured in phenol red–free RPMI 1640 supplemented with 10% charcoal-stripped FBS.

Nucleic acids

Small interference RNA for *TP53* was purchased from IDT as predesigned siRNA: sense strand 5′rCrCrArCrCrArUrCrCrArCrUrArCrArArCrUrArCrArUrGT3′, antisense strand 5′rCrArCrArUrGrUrArGrUrUrGrUrArGrUrGrGrArUrGrGrUrGrGrUrA3′. Small interference RNA for *FBXW7*: sense strand 5′-rGrGrArGrUrGrGrArCrCrArGrArGrArArArUrUrGrCrUrUG C-3′, antisense strand 5′-rGrCrArArGrCrArArUrUrUrCrUrCrUrGrGrUrCrCrArCrUrCrArG-3′. cDNA of firefly luciferase was cloned into pCI-Neo vector followed by 3′ untranslated region (UTR) of *FBXW7*, which was joined by two separate PCR fragments (left fragment: 5′CTAGTCTAGAAGAGCAGAAAAGATGAATTT3′ and 5′TTAGAGGCACAGATGGCTCA3′, right fragment: 5′TTGTCCCAACCCTGTACTGTA3′ and 5′CATGAAAAAACACATTTTATTGCACTTAAGTTATAAG3′) after restriction digestion with EcoRI followed by ligation. Mutant 3′UTR of *FBXW7* was constructed by the QuickChange method to change the sequence of consensus *miR-25* seed sequence of 5′UGCAAU3′ at two locations into a mutant sequence of 5′GGAUCC3′. Plasmid DNA encoding wild-type p53 (pCDNA3.1-p53wt) was described previously (6). Plasmid DNA encoding R175H mutation of p53 (DNp53) was generated by mutagenesis PCR. These constructs were verified through restriction digestion and sequencing analysis.

Lentivirus

p53 (R175H) was subcloned into the EcoRI site of FUCRW lentiviral vectors (7). This construct was verified through restriction digestion and sequencing analysis. The lentivirus was prepared and titered as described (8). LNCaP cells were spin infected at 1,800 rpm for 45 minutes at room temperature. All procedures were performed under University of California, Los Angeles, safety regulations for lentivirus usage.

Antibodies

Anti–Aurora A kinase antibody was from Cell Signaling Technology, anti-FBXW7 antibody was from Bethyl Laboratories, anti-p53 antibody and anti–c-Myc were from Santa Cruz Biotechnology, anti-MYCN antibody was from Abgent, anti-GAPDH antibody was from GeneTex, Inc.

Immunoblot assay

Cells were washed with PBS and lysed in RIPA buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) containing SigmaFAST Protease Inhibitor Cocktail (Sigma-Aldrich) for 15 minutes at 4°C. Cell lysates were centrifuged and supernatants were collected. Equivalent amounts of proteins as measured by Bradford assay were resolved on SDS-PAGE gels and transferred to PVDF membranes. The resulting blots were blocked in 5% nonfat dry milk in PBS for 30 minutes followed by incubation with primary antibody in 5% BSA overnight. Appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies and Supersignal West Femto chemiluminescent substrate (Thermo Fisher Scientific) were used to visualize antigen–antibody complexes.

siRNA transfection

Transfections were performed with negative control, *TP53*, or *Fbxw7* siRNA (IDT) using the Xfect siRNA Transfection Reagent (Clontech), according to the manufacturer's protocol.

Quantitative RT-PCR

Total RNA or miRNA was extracted from cells using the RNeasy Mini Kit (Qiagen) per the manufacturer's instructions. Conversion to cDNA was achieved through the PrimeScript RT Master Mix (Takara). Quantitative RT-PCR was carried out using SYBR Premix Ex Taq II (Takara), 0.4 μmol/L oligonucleotide primers, and 0.1 μg cDNA. All primer sets for quantitative RT-PCR were illustrated in Supplementary Table S1. miRNA quantification was performed using the miRCURY LNA Universal RT microRNA PCR Starter Kit (Exiqon). Relative fold change in mRNA levels was calculated after normalization to β-actin using the comparative C_t method (9).

IHC

For immunohistochemical analysis of p53 and Aurora A kinase, tissue sections were deparaffinized with xylene and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes. Heat-induced antigen retrieval (HIER) was carried out for all sections in 0.01 mol/L citrate buffer, pH 6.0, using a vegetable steamer at 95°C for 25 minutes. Mouse monoclonal anti-p53 antibody, clone 1801 (EMD, OP09-100UG) was diluted with BSA to a concentration of 1:50 and applied to the sections. Incubation was for 45 minutes at room temperature followed by anti-mouse secondary antibody (MACH 2 Mouse HRP-Polymer; Biocare Medical; MHRP520L) incubation for 30 minutes at room temperature. Rabbit monoclonal Aurora kinase A antibody (Abcam; 1800-1) was diluted with BSA to a concentration of 1:50 and applied to the sections. Incubation was for 1 hour at room temperature followed by anti-rabbit secondary antibody (Dakocytomation Envision System Labelled Polymer HRP anti rabbit, Cat.# 4003) incubation for 30 minutes at room temperature. Diaminobenzidine was then applied for 10 minutes at room temperature to visualize p53 and Aurora Kinase A. Sections were counterstained with hematoxylin, dehydrated through graded alcohols, and coverslipped. Immunohistochemical semiquantitation was performed using the Quick score (Q) method (10). Results are scored by multiplying the percentage of positive cells (P) by the intensity (I) (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining). Formula is defined as $Q = P \times I$; maximum = 300.

Immunofluorescence double staining

Slides were deparaffinized with xylene and rehydrated through graded ethanol. HIER was carried out in 0.01 mol/L citrate buffer, pH 6.00, using a vegetable steamer at 95°C for 25 minutes. Sections were permeabilized for 10 minutes with 0.25% Triton X-100 and rinsed with PBS. Blocking was done with 2% BSA for

30 minutes at room temperature. Primary antibody mixtures (Aurora Kinase A 1:100 BSA + p53 1:25 BSA) were applied for 1 hour at room temperature. Slides were rinsed with PBS, and the secondary antibody mixture (goat anti–Mouse-Alexa Fluor 488 + goat anti–rabbit-Alexa Fluor 568, both 1:500 BSA) was applied for 1 hour at room temperature. Slides were rinsed with PBS and coverslipped using VECTASHIELD HardSet Mounting Medium with DAPI (Vector, H-1500).

Statistical analysis

Statistical analyses were performed using the Student *t* test with the Excel 2013 software. Error bars indicate SD calculated from three independent experiments.

Results

P53 mutation leads to increased expression of *miR-25* in prostate cancer cells

We previously demonstrated that the quiescent NE cells in prostatic adenocarcinoma contain wild-type p53, whereas the rapidly proliferating NE tumor cells of SCNC often contain mutated p53 (3). We proposed that p53 mutation may play a critical role in the development of aggressive behavior of prostatic SCNC, but the detailed mechanisms were unclear. P53 can regulate miRNA expression in cancer cells (11). In glioblastoma cells, for example, p53 has been reported to repress the expression of *miR-25* and *-32* (12). Thus, it is quite interesting whether there is also a relationship between p53 expression/function and the expression of miRNAs, such as *miR-25* and/or *-32*, and the interaction then contributes to the biologic behavior of prostatic SCNC. We therefore tested this hypothesis in prostate cancer PC-3 and LNCaP cells. Our previous study has shown that LNCaP cells are typical prostate adenocarcinoma cells, and PC-3 cells are characteristic of SCNC (13). In addition, LNCaP cells express wild-type p53 protein, and PC-3 cells contain truncated p53 mutation, which leads to the absence of p53 protein expression.

We expressed either wild-type p53 protein in PC-3 cells, or mutant p53 that is defective in DNA binding (R175H) in LNCaP cells. We found that expression of wild-type p53 protein repressed *miR-25* expression in PC-3 cells, and expression of R175H mutant p53 protein increased *miR-25* level in LNCaP cells, both with statistical significances (Fig. 1A). However, we noticed that expression of wild-type p53 protein in PC-3 cells did not cause obvious changes in the expression of *miR-32* (data not shown). In prostate NE cancer cell line NCI-H660 that contains wild-type p53, we also observed that knockdown of p53 by siRNA or expression of the dominant-negative p53 mutant both resulted in enhanced *miR-25* expression (Fig. 1B).

We further examined whether the changes of *miR-25* level in these cells were associated with potential regulation of cell cycles induced by changes of p53 status. We found that expression of dominant-negative p53 (DNp53) did not cause obvious changes of cell cycle distribution in LNCaP cells, although *miR-25* level changes observed. In PC-3 cells, however, expression of wild-type p53 (WTp53) did induce a change in G_2–M transition 24 hours later after transfection of mammalian expressive WTp53 construct. Interestingly, we observed statistically significant reduction of *miR-25* levels in cells at this time point (Supplementary Fig. S1).

Taken together, these results suggest that p53 can regulate *miR-25* expression in prostate cancer cells, whereas mutant p53

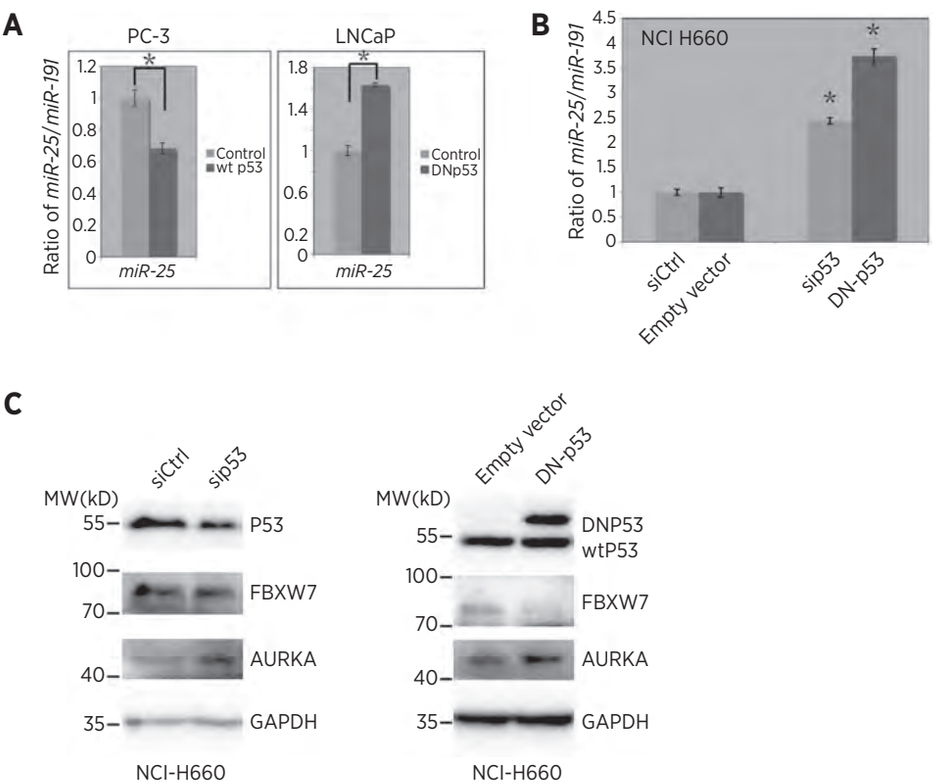


Figure 1.
p53 regulates *miR-25*, FBXW7, and Aurora kinase A. A and B, *miR-25* expression changes in response to p53 expression and mutation status. Plasmids encoding wild-type p53 (left) or mutant p53 (right) were transfected together with GFP-expression plasmid into PC-3 or LNCaP cells for 48 hours. GFP-positive cells were sorted and RNAs were then isolated for quantification of *miR-25* using qRT-PCR. Two independent triplicate experiments were performed, and results are presented as mean ± SD. The ratio of the *miR-25* over *miR-191* as an internal control was plotted (A). siRNA for p53, or lentiviral vector for dominant-negative p53 was introduced into NCI H660 cells for 48 hours, and *miR-25* was quantitated as in A (B). Asterisks, significant differences ($P < 0.05$). C, immunoblot analysis results showing the changes of proteins in response to p53 regulation for 48 hours in NCI H660 cells.

or loss of p53 functions can cause elevated expression of *miR-25* expression.

P53 mutation leads to decreased expression of FBXW7 and overexpression of Aurora kinase A

miR-25 has many potential targets including *FBXW7* and *Wwp2* (WW domain containing E3 ubiquitin protein ligase 2) (14). Indeed, we observed that overexpression of *miR-25* could lead to reduced levels of both *FBXW7* and *WWP2* in PC-3 cells, as well as in NE1.8 cells, a variant of LNCaP cells that resemble NE cells (Supplementary Fig. S2; ref. 5). *FBXW7* encodes an E3 ubiquitin ligase whose substrates include several positive cell cycle regulators, such as MYCN (15), MYC (16, 17), Cyclin E (18, 19), and Aurora kinase A (20, 21). Of them, Aurora Kinase A is overexpressed in prostatic SCNC and may play important roles in the development of aggressive prostate tumor (4). We thus further tested whether p53 mutation or loss of function could cause changes of Aurora Kinase A expression via regulation of *miR-25* and FBXW7. Indeed, as shown in Fig. 1C, we found that knocking down p53 with siRNA in NCI-H660 cells resulted in increase of Aurora kinase A expression. And expression of DNp53 in NCI-H660 led to a change of Aurora kinase A expression similar to p53 knockdown. In addition, we observed reduced expression of FBXW7 in NCI-H660 cells with these manipulations of p53 expression.

Next, we determined if change of FBXW7 expression would affect the protein level of Aurora kinase A. In NCI-H660 and PC-3 cells, we found transfections of *Fbxw7* siRNA decreased the expression of FBXW7 protein, and increased levels of Aurora kinase A. Similar results were also observed in NE1.8 cells (Fig. 2). In these cells, silencing of *Fbxw7* also resulted in elevated protein levels of its targets C-MYC and MYCN. Thus, these results suggested that FBXW7 may also function as the ubiquitin E3 ligase targeting Aurora kinase A.

***miR-25* mediates mutant p53–induced expression of Aurora A kinase**

We next determined the potential role of *miR-25* in the regulations of Aurora kinase A upon loss of p53 function in these prostate cancer cells. For this, we cotransfected p53 siRNA with *miR-25* inhibitor into LNCaP cells. Figure 3A and B showed that *miR-25* inhibitor prevented p53 knockdown–induced downregulation of FBXW7 and upregulation of Aurora kinase A in these LNCaP cells, indicating that loss of function of p53 may regulate Aurora kinase A expression through a linear pathway involving *miR-25* and FBXW7. To further clarify the roles of *miR-25* in this process, we transfected *miR-25* into NE1.8 cells. As expected, our results showed that overexpression of *miR-25* reduced FBXW7 level and increased Aurora kinase A protein expression (Fig. 3C).

To determine whether *miR-25* can regulate *FBXW7* expression through its likely target in its 3′UTR region, we generated a reporter construct where coding sequence for firefly luciferase was followed by a 3′UTR sequence of *FBXW7*, or by a mutant 3′UTR sequence of *FBXW7* where the target sequences of *miR-25* were destroyed. As shown in Fig. 3D, exogenous expression of *miR-25* caused approximately 50% reduction of the luciferase activity with the wild-type 3′UTR of *FBXW7*, whereas no obvious changes of luciferase activity was observed with the mutant 3′UTR in response to the miRNA expression. Thus, our results indicated that *miR-25* could directly regulate *FBXW7* expression through the miRNA binding site in its 3′UTR.

We also tested the potential roles of *miR-25* in the biologic behaviors of SCNC PC-3 cells. Our results showed that inhibition of *miR-25* in PC-3 cells attenuates cell proliferation and reduced invasion capability (Supplementary Fig. S3).

Taken together, our results suggested a likely signaling pathway through which p53 mutation induces upregulation of *miR-25* and downregulation of FBXW7, eventually leading to the overexpression of Aurora kinase A, which may promote rapid proliferation of the NE tumor cells of SCNC.

Coexpression of nuclear p53 and Aurora kinase A in human SCNC tissue

We further verified the coexpression of p53 and Aurora Kinase A in 12 cases of human prostatic SCNC and an equal number of cases of prostate adenocarcinoma. Our IHC study showed that, eight of the 12 prostatic SCNC cases had positive nuclear p53 staining, which usually results from mutation of p53 with increased p53 protein stability. Nine of the 12 cases also showed overexpression of Aurora kinase A, of them, 6 cases were positive for both nuclear p53 and Aurora kinase A overexpression (Fig. 4A). We also noticed that all 12 cases of adenocarcinoma were negative for both p53 nuclear staining and Aurora kinase A overexpression. Furthermore, we found the coexpression of nuclear p53 and Aurora kinase A in these SCNC tissues (Fig. 4B). These results supported our hypothesis that p53 mutation might lead to the overexpression of Aurora Kinase A in prostatic SCNC.

We have shown previously that p53 mutation is common in prostatic SCNC and rare in untreated adenocarcinoma (3). Similarly, Rubin's group (22) showed that Aurora kinase A is

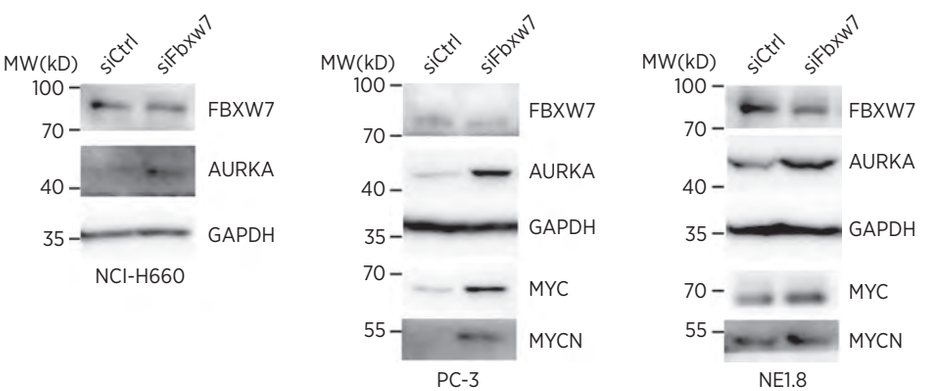


Figure 2.
Fbxw7 regulates Aurora kinase A expression. Control or *FBXW7* siRNA at 100 nmol/L was transfected into NE1.8, PC-3, or NCI-H660 cells. Aurora kinase A and FBXW7 were examined via immunoblot analysis. MYCN and MYC are included as known validated targets of FBXW7.

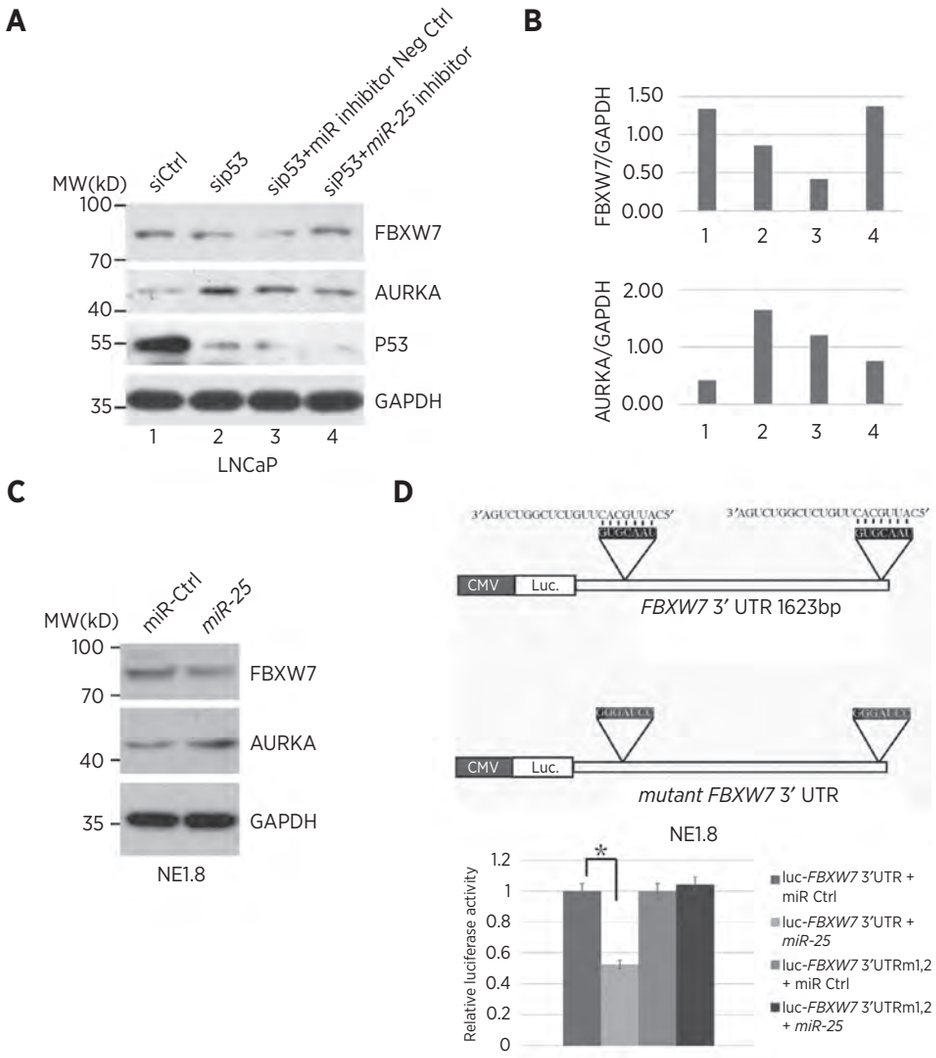


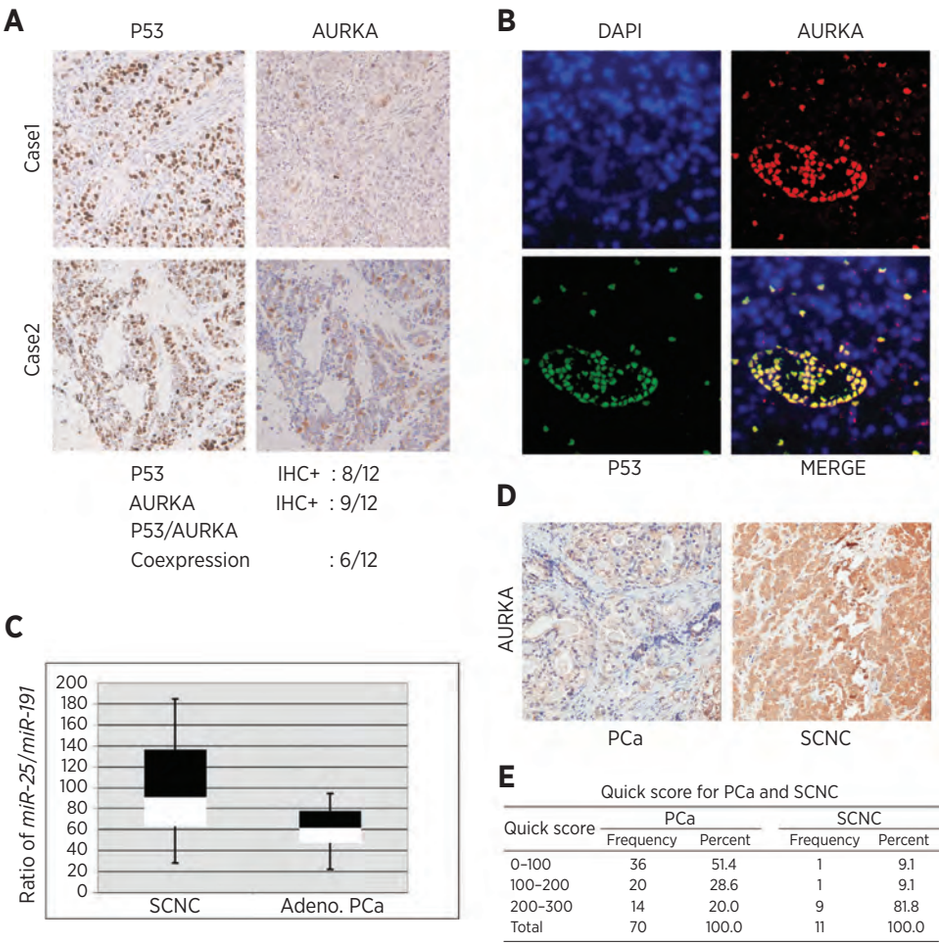
Figure 3.
miR-25–regulated Aurora kinase A is dependent on loss of p53 function. A, Western blot analysis showing that *miR-25* inhibitor eliminates the effect of p53 knockdown on regulation of FBXW7 and AURKA. B, quantification of the densitometry for Western blot bands using ImageJ 1.47t. C, the effects of *miR-25* on expressions of AUKUA and FBXW7 proteins. Control or *miR-25* mimic at 100 nmol/L was transfected into NE1.8 cells, a variant of LNCaP cells that resemble NE cells. Aurora kinase A and FBXW7 were examined via immunoblot analysis. D, a graphic representation for expression plasmids containing firefly luciferase with wild-type 3′ UTR of human *FBXW7* and the mutant 3′UTR without the target sequence for *miR-25* (top). These plasmids were transfected into NE1.8 cells together with control or *miR-25* mimic, luciferase activities were measured 48 hours later and plotted (bottom). Three independent experiments were performed, and results are presented as mean ± SD.

overexpressed in prostatic SCNC but not in adenocarcinoma. Therefore, we performed analysis for *miR-25* expression between the two types of tumors. In this study, total RNA was isolated from the paraffin section of the cancerous area from 12 cases of SCNC and an equal number of adenocarcinoma cases. The levels of *miR-25* were measured and normalized to *miR-191* as the internal control, and the ratios of these two miRNAs were then plotted. The box-and-whisker plots showed that almost half of the cases of prostatic SCNC have higher levels of *miR-25* expression when compared with prostate adenocarcinoma (Fig. 4C). Protein overexpression of Aurora kinase A in human prostatic SCNC was confirmed by IHC (Fig. 4D and E). Thus data from the human prostate cancer tissues also support the notion that p53 mutation in SCNC may lead to a higher *miR-25* expression that contributes to the higher expression of Aurora kinase A.

Discussion

Prostatic small cell carcinoma is an underdiagnosed entity because patients with widely metastatic disease after hormonal therapy usually do not undergo biopsy or resection for histologic diagnosis. Its incidence is expected to rise after the recent approval of super-blockers of AR signaling pathway such as abiraterone and enzalutamide. In addition to a lack of effective therapy, the molecular mechanisms driving the development of SCNC remain unclear.

We recently showed that p53 mutation may be a critical molecular responsible for the aggressive behavior of SCNC (23), and the study from Rubin's group reported gene amplification and overexpression of MYCN and Aurora kinase A in these tumors (4). Aurora kinase A is an evolutionarily conserved serine/threonine kinase critical for mitotic regulation (23). It can phosphorylate multiple mitosis-associated proteins (e.g., Tacc and Ndel1), thus modulating their activities (24, 25) and orchestrating centrosome maturation, spindle assembly, and mitotic entry. Aurora kinase A can also regulate protein translation through CPEB phosphorylation (26, 27). Thus, Aurora kinase A plays important roles in cell proliferation and has been considered a potential therapeutic target for prostatic SCNC. In addition, it has been also reported that wild-type p53 suppresses the expression of *miR-25* (12), and mutant p53 or loss of p53 function resulted in increased *miR-25* expression. The relationship between mutant



E

Quick score for PCa and SCNC

Quick score	PCa		SCNC	
	Frequency	Percent	Frequency	Percent
0–100	36	51.4	1	9.1
100–200	20	28.6	1	9.1
200–300	14	20.0	9	81.8
Total	70	100.0	11	100.0

Figure 4.
Expressions of p53, Aurora kinase A, and *miR-25* in human prostatic small cell carcinoma. A, representative images show the IHC staining with anti-p53 and anti–Aurora kinase A antibodies in human prostatic small cell carcinoma B, representative images showing the coexpression of nuclear p53 and Aurora kinase A in these human prostatic small cell carcinoma using immunofluorescence double staining. C, *miR-25* was quantitated in small cell carcinoma or adenocarcinoma into box-and-whisker plots, the ratio of the *miR-25* over *miR-191* as an internal control was plotted. D, differential expression of Aurora kinase A in human prostatic adenocarcinoma or small cell carcinoma. Representative image showing the results for IHC staining with antibody for Aurora kinase A in these different types of prostate cancers (PCa). E, semiquantitation of AURKA IHC staining was performed using the Quick score method.

p53 and overexpression of Aurora kinase A in SCNC is thus quite interesting. In present study, we show that expression of mutant p53 protein leads to enhanced expression of Aurora kinase A in prostate cancer cells, and this was most likely mediated by increased *miR-25* expression and decreased expression of FBXW7 subsequent to p53 mutation.

miR-25 is a well-studied oncogenic miRNA. It is 22 nucleotides long, localized in the minichromosome maintenance protein-7 (MCM7) gene, and transcribed as part of the *miR-106b*∼*25* polycistron. It is overexpressed in several human cancers, including pediatric brain tumors (28), gastric adenocarcinoma (29), EGFR-positive lung adenocarcinoma (30), and prostate carcinoma (31), and has been reported to target different regulators of the apoptotic pathway, such as BIM (32), PTEN (31), and TRAIL (33). *miR-25* also affects Ca^{2+} homeostasis by regulating mitochondria calcium efflux through targeting the mitochondria calcium uniporter (34), causing a strong decrease in mitochondrial Ca^{2+} uptake and, likely, conferring resistance to Ca^{2+}-dependent apoptotic stimuli. We found that p53 mutation–induced *miR-25* overexpression downregulates the expression of ubiquitin E3 ligase FBXW7. FBXW7 is a potent ubiquitin E3 ligase that can degrade Aurora kinase A (35), lower level of FBXW7 thus leads to increased protein level of Aurora kinase A. In PC-3 and NE1.8 cells, we noticed that transfection with *miR-25* reduced mRNA expressions of Aurora kinase A, but significantly increased protein levels of Aurora kinase A which can be affected by exposure to cycloheximide, a protein synthesis inhibitor (Supplementary Fig. S4), suggesting that the elevated protein level of Aurora kinase A in these cells was caused by the FBXW7-induced blockage of Aurora kinase A degradation. Inactivation of FBXW7 has also been noticed to be critical for the proliferation of leukemic stem cells, and contributes to the development of leukemia (36, 37). Thus, our results suggest a potential signaling pathway that how mutant p53 regulates level of Aurora kinase A in prostate cancer cells that

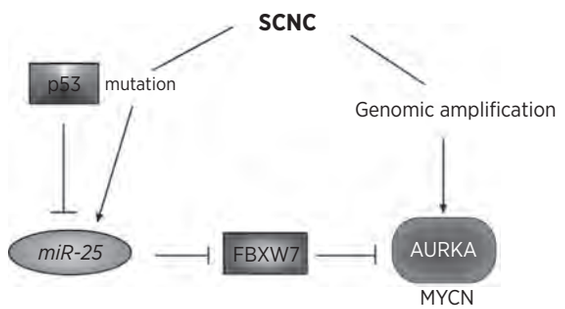


Figure 5.
A diagram depicting multiple pathways that lead to overexpression of Aurora kinase A in human prostatic small cell carcinoma.

may be correlated to rapid proliferation and aggressive behavior of prostatic SCNC.

Although our data suggest the presence of a linear pathway of p53 → *miR-25* → FBXW7 → Aurora kinase A in SCNC, there are likely other important players in the pathogenesis of prostatic SCNC. In a significant number of SCNC cases, Aurora kinase A overexpression is associated with gene amplification, which involves different genetic events. Of note, Rubin's group has shown that MYCN is also amplified and overexpressed in prostatic SCNC (4). Because expression of FBXW7 can also cause degradation of MYC, it would be interesting to study if p53 mutation also leads to MYC overexpression. In addition, Collins' group reported that downregulation of the REST transcription complex may lead to the development of SCNC (38). These diverse findings suggest that the pathogenesis of SCNC is a complex process that may involve different players and multiple signaling pathways (Fig. 5).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Sun, X. Chen, C. Liang, J. Huang
Development of methodology: Z. Li, Y. Sun, C. Liang, J. Huang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Li, Y. Sun, X. Chen, J. Squires
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Li, Y. Sun, B. Nowroozizadeh, J. Huang
Writing, review, and/or revision of the manuscript: Z. Li, Y. Sun, X. Chen, J. Squires, J. Huang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Li
Study supervision: J. Huang

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